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SUPERCRITICAL FLUIDS FOR OFF-LINE SAMPLE PREPARATION IN FOOD ANALYSIS PRIOR TO CHROMATOGRAPHY

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I. SUPERCRITICAL FLUIDS FOR SAMPLE PREPARATION

Sample preparation prior to chromatography has been an integral step of analytical method development that has received increasing emphasis in recent years. The rationale for this trend is the increasing complexity of chemical analysis which continues to place a burden on the analyst using chromatographic methods. Hence, improvements in sample preparation prior to analysis via chromatographic techniques can reduce substantially the complexity of such assays as well as reduce the attrition on columns and associated instrumentation. There are a plethora of sample preparation methods available, and this review will focus the attributes of supercritical fluids and similar compressed media as agents for this process.

There are good reasons to consider the use of supercritical fluids in sample preparation prior to chromatography, particularly since the fluid is easily removed from the sample matrix after extraction or sample cleanup. The most widely used supercritical fluid, supercritical carbon dioxide (SC-CO_2), is relatively, inexpensive, non-flammable, and environmentally-benign. This last feature has been a key factor in the development of critical fluid-based methods in the early 1990's when legislative and regulatory mandates on the use of hazardous solvents were officially promulgated [1]. Such acts as the U.S. Environmental Protection Agency's (EPA) Pollution Prevention Act, the Superfund Amendments and Reorganization Act (SARA), the Resource, Conservation, and Recovery Act (RCRA) and Montreal protocols were designed to reduce or eliminate the use of carcinogenic or environmentally-adverse compounds, such as chlorinated solvents; aromatics (i.e., benzene); and fluorinated hydrocarbons. Thus, supercritical fluid extraction (SFE) using SC-CO_2 provides a viable alternative to using the above solvent media and potentially totally reducing the analyst's dependence on organic solvents altogether.

SFE also competes with an assortment of other, relatively new sample preparation techniques, that have been developed toward reducing the use of copious amounts of organic solvents. Practically all of these new sample preparation methods share some of the same generic features: a substantial reduction in the amount of solvent, reduction in the sample size of the sample matrix, and high sample throughput via automated, unattended operation. When one considers that classic extraction methods, such as the Soxhlet extraction technique have been used for over 90 years [2], it is somewhat surprising that newer methods have not evolved at a faster pace in the interim.

Modern supercritical fluid technology is documented in many books and reviews which cover both processing as well as analytical utilization of these unique fluids. Some interesting primers for the novice to the field are by Taylor [3], Clifford [4] and Luque de Castro, et al. [5]. Although engineering theory and applications might appear as having questionable relevency to the analytical chemist or chromatographer, nothing could be farther from the truth. The theory and fundamental principles of SFE share intradisplinary application as will be emphasized in the review the follows. Key tomes involving the fundamentals and processing utilization of critical fluids are Brunner [6], McHugh and Krukonis [7], and Mukhopadhyay [8]. In addition there are approximately 45 other major references dealing with the subject of supercritical fluids, and the author has listed these in APPENDIX I.

This review admittantly focuses on the use of off-line SFE and its variants in preparing samples prior to chromatography. There are some researchers who share the view the analytical SFE is a derivative of activity in the field of analytical supercritical fluid chromatography (SFC) [9]. Although both analytical techniques share a common physicochemical basis, analytical SFE

stands on its own merits, whether expedited in either the on-line versus off-line mode. For semantic purposes on-line SFE will refer to the technique when directly linked in tandem with an another analytical technique, such as gas chromatography (GC), Fourier-transform infrared spectroscopy (FTIR), high performance liquid chromatography (HPLC), or mass spectrometry (MS). Whereas on-line SFE, particularly when coupled with SFC preceded off-line SFE in terms of development, it is the latter technique that has seen widespread use and resulted in commercial instrumentation [10]. Unfortunately such on-line SFE techniques require a relatively on level of operator training to facilitate their use routinely in an analytical laboratory; consequently there is a paucity of instrumentation that can be purchased outright for conducting on-line SFE. Despite these limitations, the reader is encouraged to read the volume by Ramsey [11] to appreciate the merits of on-line SFE methodology.

In this review, some of the basic principles of supercitical fluids (SFs) will be presented, including their optimization during analytical SFE. Types of extraction and instrumentation will also be discussed, as well as the preparation of the sample prior to SFE, and collection of the extracted analyte after SFE. Integration of sample cleanup during and after SFE will be covered with an emphasis on handling the problem of coextractives which can plague chromatographic separation of the extracted analytes. Coupling selective reaction chemistry (derivatization) in SFs is another viable alternative toward easing the burden of sample preparation for the analyst. Such supercritical fluid reactions (SFRs) can be integrated directly in-situ with the sample prep step, directly on-line with chromatographic instrumentation. Finally, selected applications of critical fluids for sample preparation prior to chromatography will be cited with a bias towards our own method development in trace toxicant and lipid analysis [12].

II. SUPERCRITICAL FLUID EXTRACTION (SFE)

A. Basic Principles of SFE

The supercritical fluid state for any substance maybe defined as existing above a specific temperature, known as the critical temperature, T_c, and a specific pressure, P_c, the critical pressure. Its relationship to other states of matter in the case of carbon dioxide is shown in Fig. 1. Here both T_c and P_c define a critical point on a pressure-temperature diagram, and correspondingly define a critical density, which in the case of CO_2 is approximately 0.45 g/cc [13]. One of the practical implications of these defined properties is that CO_2 cannot be converted to its liquid state no matter what pressure is applied, as long as it is held above T_c. As we shall see, this confers some unusual and exploitative properties on critical fluids that can be used to advantage in performing SFE. So-called "near critical fluids " have also been used to advantage, by operating the extraction in a temperature range slightly below the critical temperature (usually in the range of 0.85-0.95 in terms of the reduced temperature, $T_r = T/T_c$.

When a fluid meets the above criteria, it exhibits physical properties that are intermediate between those of a gas or liquid, and its density can be changed by varying the applied pressure on the fluid. Therefore, when a fluid is in a state of high compression it takes on a high density, approximating those associated with liquid solvents. Under such conditions, the supercritical fluid has the capability of dissolving a variety of materials, just as liquids do. Also, to a more limited extent, the selectivity of a supercritical fluid can be changed by altering its density, akin to changing liquid solvents in conventional extraction. However at high densities, the extraction selectivity of supercritical fluids is lost and their molecular specificity approximates that found for non-polar to moderate polar solvents (in the case of CO_2).

To illustrate the above concepts, the changes which occur for a model solute, naphthalene in SC-CO_2 are shown in Fig. 2. Here, a solubility of 5.2 mole % is found for naphthalene at a pressure of 300 atmospheres and a temperature of approximately 55°C, parameters which correspond to conditions used in the extraction stage of SFE (E_1). Separation of the naphthalene from the compressed CO_2 after SFE can then be affected in one of two ways as depicted in Fig. 2. The separation of the solute from the SC-CO_2 can take place at a constant pressure (300 atm) while the temperature is lowered to 20°C, thereby affecting a solubility change of 4.0 mole % (S_2). However an even larger change in naphthalene's solubility in SC-CO_2 can be achieved by reducing both the pressure and temperature at the separation stage (S_2), by adjusting the pressure to 90 atm and temperature to approximately 45°C. Under these conditions only 0.1 mole % of naphthalene is left in the SC-CO_2 phase.

The concept of "threshold pressure" with respect to supercritical fluids, has it origins in the early studies of "dense gas chromatography" [14], the historical forerunner to SFC. This is defined as the pressure (at a specific temperature) at which the analyte can first be solubilized and detected in the extraction fluid. Threshold pressures are dependent on the detection method employed for estimating the initially solubilization of the solute in the SF, and can vary over magnitudes of concentration [15], depending on whether the detection technique is a element specific GC detector, a TLC spot test, or a gravimetric balance. Threshold pressures will also be dependent on the sample matrix to some extent for a common solute, hence this factor should also be specified when quoting threshold pressures [16]. Despite these factors, the threshold pressure is a useful concept since it allows the analyst to know the minimum pressure conditions required for extracting his analyte from a given sample. Threshold pressures tend to have a weak

dependence on temperature and molecular weight, and can be estimated for SFE and SFC using the guidelines developed by King [17].

A solute's maximum solubility in a SF may or may not be of importance to the analyst depending on the specific analysis problem being considered. For example, for trace analysis purposes, there is usually sufficient solute solubility in the supercritical fluid based just on solubility considerations. However if one is analyzing the fat content of a food matrix, then conditions for affecting high lipid solubilities in SFs are desired, in order to minimize extraction time. Solute solubilities can be calculated as a function of fluid density from equations of state [18,19], or estimated from solubility parameter theory [17,20]. In some cases the use of higher extraction fluid densities is desired to remove the target analyte from a very adsorptive sample matrix.

When solutes are dissolved in supercritical fluids they exhibit higher diffusivities than they do in liquids, thereby facilitating rapid mass transfer of the solutes from the sample matrix. Corresponding mass transport properties or dimensionless also take on intermediate values between those of a dilute gas and liquid, and exhibit a dependence on fluid density. The end result of these trends is that faster extraction fluxes can be achieved using SFs, corresponding to a substantial reduction in extraction time. Indeed, for effective analytical SFE to occur, the triad noted in Fig. 3: the analyte's solubility, diffusion, and interaction with the sample matrix, must all be considered in designing optimal extraction conditions.

The rate of solute removal from a sample matrix using a SF is similar to those found whe using liquid extraction solvents, except the time required is usually less. This is illustrated in Fig. 4 for the extraction of fat from a ham matrix. Initially the extraction kinetics are governed

by the solubility of the lipid in SC-CO_2, that is to say, there is an approximately linear removal of lipid into the SC-CO_2 from the ham matrix [21]. This then gives way to a transition region in which the removal of the fat becomes rate limiting, followed by an asymptotic approach to the final lipid content with passage of the extraction fluid (SC-CO_2). Such extraction curves have been modeled by several investigators and generalized in a "hot ball" kinetic model by Bartle et al. [22].

Knowledge of the extraction kinetics in SFE is important since it determines the time and quantity of extraction fluid required to complete the extraction. In addition, such extraction rate curves can be diagnostic, suggesting that if the extraction takes to long, that the addition initially of a static extraction sequence may be beneficial. SFE can be facilitated using both the dynamic and static modes of extraction, either individually or coupled in a step-wise sequence. In the dynamic extraction mode, the fluid is conveyed continuously through the sample matrix, while in the static mode, extraction fluid is pumped into the vessel containing the sample, and held for a pre-determined time, prior to initiating dynamic extraction of the sample. In certain SFEs, just a single static extraction step is sufficient to yield the desired extract [23].

B. Types of Extraction and Instrumentation

Instrumentation for analytical SFE evolved from home-built equipment, frequently constructed on a modular basis using several types of fluid delivery options (pumps, compressors, etc.) and pressure reduction/collection devices crafted from commercial vales/regulators, or even silica capillary tubing. These basic units were improved upon by instrumentation companies, resulting in the commercial instrumentation of today which feature unattended operation, multi-sample

capability, and several analyte collection options. Table 1 lists some of the desired features for performing analytical SFE. Most modern instrumentation is capable of conducting SFE up to pressures of 680 atm (10,000 psi), temperatures in excess of 100°C, and flow fluid rate ranges to 10L/min (CO_2 at STP). Sample size, a subject that was initially quite controversial in the early development of analytical SFE, is typically 1-10 g on commercial instruments, but options do exist to easily extract up to 50 g on certain instrumentation or home-built equipment. Collection options will be discussed in Section II-E while typical automated instrumentation will be cited later in this section. Depending upon how instrumentation is configured it is possible to process in excess of 6 samples in one run, and up to 24 on one particular instrument. Cosolvent addition capability, which is highly desired in many applications, is available as an add-on feature.

Fig. 5 illustrates a basic, home-built extractor design which has been successfully utilized in our laboratory for over 20 years [24]. The unit consists of a gas booster unit which delivers pressurized gaseous CO_2 from a cylinder (A). The booster pump can easily provide extraction pressures of 680 atm and high flow rates for processing larger samples. The carbon dioxide is delivered without heat tracing to a oven enclosure (dotted line), and can be diverted downwards or upwards into a vertically-positioned extractor vessel using double switching valves (SV-1, SV-2). Conversion of the fluid to the supercritical state prior to extracting the sample is achieved using a generous length of coiled tubing (HC-1, HC-2). Extraction cells can be fabricated using 316 stainless steel tubing of varying lengths. Depending on the wall thickness, these extraction vessels can hold 50-70 or 100-140 mL of material for 70 or 140 MPa extractions. The extract is conveyed out of the extractor through another dual switching valve (SV-3, SV-4) into a heated micrometering valve. This valve must be heated to counteract the effects of Joule- Thomson

the first companies to provide instrumentation for off-line SFE: their Model SFX 2-10 and SFX 220. Both units deliver the extraction fluid via syringe pumps with varying capacity and pressure range, although the 5000 and 10,000 psi pump modules are normally purchased for use with SFE. Extraction cells of 0.5, 2.5, and 10 mL are offered in three different cell materials: stainless steel, aluminum, and a high temperature compatibile polymeric composition (9 mL disposible cartridges). Although the SFX 2-10 module is entirely manual in operation, the extraction cells can be sealed without the need of wrenches (hand tightened); permitting two extractions to be conducted in parallel. Depressurization of the solute-laden fluid is normally accomplished through the use of either a fixed flow rate or adjustable flow rate, heated coaxial back pressure restrictor. Control of the fluid delivery flow rate, extraction cartridge temperature, and restrictor temperature is achieved by microprocessor control. The SFX 220 is an automated version of the SFX 2-10 with automated valve operation for increase sample throughput.

Advantages of the above units is their modularity which permits the analyst to design and alter the extraction unit. Cosolvents are delivered with the aid of an additional pump through the microprocessor controller. There is considerable flexibility in collecting the extract; both neat and solvent-based collection tubes can be interfaced with the coaxial heated restrictors. The analyst also has the advantage of designing about any type of collection system with these units, including the use of sorbent-laden cartridges for on-line collection of lipids and volatiles for further sample preparation or off-line analysis.

Isco, Inc. also produces the Model SFX 3560 which allows up to 24 samples to be extracted sequentially. This module can operate unattended overnight through an interactive 80 X 24 microprocessor display, allowing both high sample throughput as well as automated

method development. Programmable fluid "wash" cycles between each SFE is an integral part of the instrument's operation, and both static and dynamic extraction modes can be performed on individual samples up to 10,000 psi and 150°C. Extract collection is accomplished using empty or solvent-filled vials using an automated, feed back controlled heated restrictor to prevent icing. To aid in extract collection, the 20 mL glass tubes used for collection can be cooled as low as -20°C as well as be pressurized above ambient conditions.

Applied Separations, Inc. (Allentown, PA) offers several extraction units based upon protoypes developed in the United States Department of Agriculture (USDA) laboratories [24] that offer considerable flexibility with respect to sample size and experimental design. These units can be purchased as single modules (the Spe-ed 2 or 4) having the capablity of extracting 2-4 samples in parallel. SFEs can also be conducted at high temperatures (250°C) and up to 10,000 psi (680 bar) with these units. Extractor vessel sizes can range from several mLs to one liter if required. With these units, the analyst has considerable choice with respect to the type of extract collection system that can be employed with the Spe-ed units.

Leco Corporation (St. Joseph, Michigan, USA) produces a Total Fat Analyzer designated the Model TFE 2000. Although lacking the modularity of the above-described instrumentation, the unit is carefully designed (and marketed) for total fat/oil determination using SC-CO_2 as the extraction agent. The unit accomodates 10 mL extraction cells and operates up to 10,000 psi and 150°C. Flow rates from 0-5 L/min (expanded CO_2 flow rate) are regulated using heated variable restrictors. A single module will accomodate up to 3 extraction cells, but separate units can be "piggybacked" to allow extraction of up to 24 samples in parallel.

With all of the above instrumentation, CO_2 is the preferred extraction fluid for reasons

previously cited. For total fat or oil extractions, high purity SFE-grade CO_2 is not always required, however the impurity and moisture levels in various industrial grades of CO_2 can accumulate and adversely effect instrumental performance. This accumlation of contaminents is of particular concern when analyzing for trace components using SFE, since the extraction step will tend to concentrate these contaminents in the collection stage. Such an accumulation of contaminates can ultimately interfere with the off-line analysis techniques, such as gas or liquid chromatography. Ultra-high purity grades of CO_2 (SFE and SFC grade-CO_2) are available from several vendors of laboratory-grade gases, but they are relatively expensive. Alternatively, fluid purification schemes, such as those reported by Hopper et al. [28], or CO_2 purification using a microporous ceramic oxide catalyst [29], can be employed to purify even welding grades of CO_2.

When utilizing analytical SFE, one should avoid the use of helium headspace-padded CO_2 cylinders. This technique, originally developed to avoid the use of circulating coolers with fluid pumps, introduces small quantities of helium into the CO_2 phase in the pressurized cylinder. Several investigators [30,31] however have shown that the presence of He admixed with CO_2 can reduce the solubility of solutes relative to their solubility in neat CO_2. For example, the presence of helium in CO_2 will reduce the solubility of lipids in SC-CO_2 from 33-50%, depending on the chosen extraction pressure and temperature. Therefore use of such CO_2 sources can lead to lower analyte recoveries from sample matrices and hence inconsistent analytical results.

C. The Sample Matrix and Its Preparation for SFE

The choice of sample size for any analytical determination or preparation is perhaps more crucial than many analysts realize, and this applies equally as well when using SFE. In recent years

there has been an increasing trend toward smaller sample sizes due to two factors: improved sample comminution methods and the desire to have smaller analytical instrumentation in the laboratory environment, i.e., smaller "footprints" on the benchtop. The latter factor to some extent guided the design of the initial SFE instrumentation offered commercially, which as noted previously, accomodated an average sample size of approximately 10 g. Sample sizes smaller than this puts a premium on assuring sample homogeneity through mixing, grinding, and similar processes. However, such sample treatments must not alter the sample matrix via mechanical or thermal maens, so that even the 'homogenized" sample is no longer representative of the original whole sample.

An application of analytical SFE where sample size becomes important is in the SFE of aflatoxins from corn and similar seed/grain matrices, an extraction which usually requires the use of a cosolvent to achieve suitable analyte recoveries [32, 33]. Aflatoxins in this particular case are generated on the corn matrix from infestation of a fungi, *Aspergillus flavus*, and evolve and spread from a specific site, leading to a potential maldistribution of the target analyte on a single kernel of corn, throughout a corn ear, or resulting in "hotspots" within a corn elevator. Therefore obtaining a representative sample for SFE or any other extraction/sample preparation procedure is difficult, considering that the toxicity of the analyte does not make it very amenable to many standard homogenization techniques. Table 2 shows the recovery results of aflatoxin B_1 from different quantities of the same corn sample for both solvent and supercritical fluid extraction. The SFE result in this case was obtained on a 3 g sample. Obviously comparison of the SFE-extracted sample to a 50 g solvent-extracted sample (CB method and 50g-Method 1) could lead to a low recovery in the SFE case. However, comparison of the SFE result to a 3 g solvent-

extracted sample indicates that both extraction techniques produce similar recoveries, if a 3 g samples is representative of the true aflatoxin

There is little doubt, based on statistical sampling theory [34], that using a larger sample size in any type of extraction yields more precise results. An example of this is shown in Figure 7 for the determination of the fat content of potato chips using SFE. Here one obviously sees the tradeoff between sample size and precision of analysis. These results have ramifications in terms of comparing SFE with traditional extraction methods, which are usually based on much larger sample sizes. Nevertheless, with proper homogeneity, even SFE using small samples sizes has yielded good precision indices [35]; comparable with those found via established methodologies.

Sample preparation prior to SFE consists of the following: comminution of the sample if needed, controlling the amount and effect of water on the sample matrix, and dispersion of the sample matrix prior to SFE. Mechanical grinding of the sample prior to SFE to decrease the particle size will usually increase the mass transfer of the target analyte, resulting in a faster SFE [36]. Likewise, the use of sorbent mulling, e.g., matrix solid phase dispersion (MSPD) [37] can effectively disrupt the sample matrix, aiding recovery or retention of the desired analyte from the sample matrix.

Since samples intended for SFE are often placed in tubular-configured extraction cells, it behooves the analyst to try and produce a particle size which will yield an optimal extraction. In this regard, chromatographic theory may be applicable for optimizing the particle diameter of the sample to column diameter ratio [38]. Although SFE does not usually occur under conditions of turbulence in a packed bed, SFE can be modeled as a chromatographic process [39], a factor which should aid the analyst in optimizing the particle diameter consistent with the dimensions

of the extraction cell. This will assure mixing of the extraction fluid with the sample matrix and reduce conditions which lead to channeling in the bed, and subsequently, incomplete extraction.

In some instances, sample grinding can be detrimental in SFE, particularly when the analytes of interest are located on the surface of the sample particle. This is particularly true when dealing with samples containing potential unwanted coextractives, which may interfere in the analysis and require cleanup of the supercritical fluid extracted sample. In this specialized case, SFE on the neat sample may prove more effacacious. For example, on-line SFE of the seeds of the desert smoke tree, *Dalea spinosa*, yielded extract compositions that depended on the comminution of the sample [40]. Grinding the seed sample allowed the SFE of higher molecular weight components, i.e., triglycerides, which were not desired. Therefore, in this case, extraction on the unground sample is to preferrred versus sample grinding.

The use of pelletized celite, i.e., Hydromatrix, when mixed with a sample matrix solves many of the sample preparation problems in SFE [41]. This patented concept uses large particle size diatomaceous earth to disperse many sample types very effectively, and is marketed by SFE instrumentation companies under various product designations. An addition benefit of using Hydromatrix is that it will also adsorb approximately twice its weight in water, and hence can be used to prepare high water-containing samples for SFE successfully.

D. The Problem with Coextractives and Water

As noted previously, some degree of fractionation can be achieved in SFE by adjustment of the fluid density, thereby allowing proximate separation of solutes which differ considerable in their molecular weight and/or volatility. Because of the propensity of SC-CO_2 to dissolve fats or oils,

lipid-type compounds are frequently extracted along with the desired analytes, particularly from biological sample matrices. Such samples along with environmental matrices also contain water which can also be coextracted during SFE.

Solubility data on lipid solutes in SC-CO_2 have been determined over a range of pressures and temperatures [42,43], and can be used to minimize carryover of lipids into the final extract when using SFE. Fig. 8 shows the dependence of triglyceride solubility in SC-CO_2 as a function of temperature and pressure. Note that a relatively low weight percent solubility in SC-CO_2 (5%) is found for triglycerides at 40°C and 50°C, but as the temperature is increased upwards from 50°C to 60°C, there is a pronounced increase in triglyceride solubility, particularly at higher pressures. Further increases in temperature enhances the triglyceride solubility substantially and can result in solubilities that exceed 40 wt. % in SC-CO_2 at 700 bar. Such solubility trends in SC-CO_2 have been employed routinely to perform oil and fat extractions using SFE [44]. Based on the density dependence of fat solubility in SC-CO_2, Gere [45] has defined a "fat band" of fluid densities for SFE which should not be exceeded to prevent coextraction of lipid moieties.

Water likewise has a finite solubility in SC-CO_2 as shown in Fig. 8 [46], a factor which can lead to problems in SFE, or its potential exploitation in assuring a more efficient extraction. As noted in Fig. 9, over the range of pressures and temperatures commonly employed in SFE, there is a monotonic solubility of water as a function of pressure in SC-CO_2. Despite this low solubility level of water in SC-CO_2, water can be problematic, leading to irreproducible results, contamination of the extract, and problems associated with restrictor function and/or collection devices.

A convient way of suppressing the is effect is to add a dessicant to the sample matrix to

adsorb the water. Alternatively, a quantity of dessicant can also be added to the extraction cell downstream of the sample matrix, but this can lead to problems in facilitating the extraction. The choice of dessicant is critical in order to avoid caking of the sample matrix which could impede the extraction process. The choice of drying agent can be made by consulting the study of Burford, et al. [47] in which several common dessicants were evaluated with respect to their efficiency in analytical SFE. Considerable success has been achieved using Hydromatrix which embraces not only many of the properties of the ideal sample dispersant, but aids in the retention of modest water levels in moist samples. Control of water during the SFE is also important for minimizing the plugging of restrictors, since with the attendant Joule-Thompson effect which is present during the expansion of SC-CO_2 to atmospheric pressure, can result in ice formation at the restrictor orifice, resulting impedance of fluid flow.

The role of water in SFE can be twofold; that of synergist in facilitating extraction or as an inhibitor in sterically blocking contact between the analyte and the extraction fluid. It has been noted in engineering scale studies of SFE [48] that water can modify the morphology of the sample matrix, leading to improved mass transport of the extract (analyte) out of the sample. The most oft-cited case of this phenomena is the extraction of caffeine from coffee beans which can only be effectively accomplished with a moist bean matrix. The natural presence of water in such a sample matrix also facilitates it use as a in-situ "cosolvent", since it presence during the extraction of polar analytes can lead to enhanced extraction recoveries [49].

Conversely, large quantities of water in the sample matrix, which is frequently the case during the SFE of foods, natural products, and biological tissue, can inhibit extraction due to a reduction of contact between the fluid and analytes. Nowhere is this more prevalent than in the

extraction of lipids from moist tissue samples [50]. Fig. 10 illustrates the dramatic effect of dehydrating the sample prior to SFE with carbon dioxide on a ham sample containing over 70 wt% water. Quantitative recovery of total fat content, which is desired for pesticide residue analysis, is clearly inhibited by the presence of such a relatively large quantity of water. Gentle dehyration of the sample in a oven prior to SFE, or freeze drying , will rapidly facilitate the removal of water, and ultimately fat (Fig. 10), reducing both the time and mass of CO2 required for the extraction.

E. Collection of the Extracted Analyte

As noted by Taylor [51], optimization of the collection method for the resultant extract from SFE is often neglected, resulting in incomplete analyte recoveries that are falsely associated with an incomplete SFE. There are several techniques for collecting or trapping the extracted analytes in analytical SFE and each is effected by temperature. The most often utilized options are open vessel, liquid and sorbent trapping of analytes. Some empirical and experimental-based studies have been reported by various researchers, particularly Taylor and colleagues [52], who have studied the impact of various experimental parameters on analyte trapping efficiency.

Collection in a empty vial or vessel has been successfully practiced by a number of investigators and is particularly appropriate for bulk extraction of fat and similar exhaustive extractions. It is also applicable however, for the extraction of trace levels of analytes, such as pesticides, but larger collection vessels are required for capturing such trace analytes in order to minimize their loss. Avoidance of entrainment of analytes in the escaping fluid stream can be minimized by adding a glass or steel wool or ball packing to the empty container. The chosen

material should be chemically inert, provide a high surface for condensing the analyte from the rapidly expanding fluid, but allow for the ready desorption of the analyte after completion of the extraction. A novel scheme for inserting a open collection vessel prior to a sorbent trap at allow for trapping of both non-volatile and volatile constituents from food matrices is shown in Fig. 11 Here, the initial collector serves to capture coextracted lipid constituents, while volatile species are isolated downstream on sorbent-filled tube (B). In addition it is also possible to design a collection scheme that permits the concentration of volatile species in a coextracted oil under pressure .

King and Zhang [53] have modeled solute trapping in a open vessel in terms of the retention efficiency of the analyte being collected and shown that trapping effciency is related to the relative vapor pressures of the solute (analyte) and the solvent (supercritical fluid). Since CO_2 upon decompression has a large fugacity, it is not unusal for the ratio of solvent/solute vapor pressures to exceed 10^3. Despite this favorable phase separation, it is best to use a collection vessel packed with a surface area material,i.e., glass beads or wool in order to avoid entrainment of target analyte in the escaping fluid [54].

Taylor and coworkers have studied collection efficiency using both neat and modified collection solvents [55-57], for both model test solutes and fat soluble vitamins. Similarly, Langenfeld, et al. [58] measured the effect of collection solvent parameters as well as extraction cell geometry for over 65 different compounds in 5 different solvents. More recently, Vejrosta, et al. [59] have reported optimizing the collection device, for low boiling compounds having a vapour pressure similar to the collection solvent, where significant analyte losses can occur.

Analyte collection in a sorbent filled collection device has been utilized in analytical SFE

for many years, and has been an integral component in older instrumentation that is no longer commercially produced (i.e., Hewlett Packard Model HP 7680 and Suprex Autoprep 44).. Successful application of this mode of collection requires an appreciation of the potential of analyte breakthrough off the collection sorbent as the extraction continues. Breakthrough characteristics for a number of common volatile compounds have been measured by gas-solid chromatography using CO_2 as a carrier gas [60], and have been shown to be considerable less then those found with helium as a carrier gas. This result is a direct reflection of the enhanced interaction between low pressure CO_2 and typical organic solutes, i.e., indicating that CO_2 is a favorable medium for extracting volatile compounds at very low pressures.

Taylor and coworkers [61-62] have conducted studies to measure the trapping efficiencies of various adsorbents with neat and modified supercritical carbon dioxide (SC-CO_2), the variance in the trapping capacity for different types of solid phases, and the effect of trap temperature on analyte recovery. Chaudot et al. [63] also studied the effect of modifier (cosolvent) content on the trapping efficiencies of analytes on various adsorbents, and showed that analyte retention was possible even when the modifier content of carbon dioxide was quite high (e.g., 10% methanol).

While faster flow rates may yield rapid extraction rates, the analyst may have to consider the tradeoff between the speed of extraction and good collection efficiencies. Experience has shown that a high fluid velocity through the extraction cell may result in too large a flow rate of expanded extraction fluid into the collection device. This can lead to lower collection effciencies and entrainment of the analytes out of the collection device into the expanding gaseous stream.

III. INTEGRATION OF CLEANUP STEP WITH SFE

Analytical SFE is capable of yielding crude fractionations by changing the fluid density, but it is rare to obtain a "clean" extract unless the sample matrix is insoluble in the supercritical fluid, or the compounds to be isolated, differ substantial in their physicochemical properties (i.e., polarity, vapor pressure, molecular weight. For example, the separation of fat from a food matrix or contaminents in a soil sample can be handled quite adequately by SFE. On the other hand, the isolation of pesticides from a food sample that contains appreciable quantities of fat or water, may be more problematic.

In some cases, a judicious choice of extraction fluid density may provide an extract that is perfectly acceptable for analysis without the need for further cleanup of the extract. SFE can be made potentially more selective then liquid extraction, since the density or solubility parameter of the fluid can be varied with extraction pressure or temperature. However, it is common place in the practice of SFE to use a sorbent, either in the cell, or after decompression to further fractionate the extract. Sorbents used for this purpose tend to be classified chromatographically as normal phase chromatography adsorbents, since SC-CO_2 is in someways analogous to non-polar solvents. Therefore SFC may be useful as a screening tool to chose the optimal sorbent for cleanup of the extract under SFE conditions.

As shown in Table 3, there are a number of ways for simplifying a supercritical fluid-derived extract. These include of course varying the pressure, temperature, and time of extraction to yield an extract containing the target analytes of interest, thereby reducing the total number of coextractives (if there are any). Other options can include changing the type of extraction fluid or fractionating the extract according to individual solute (analyte) threshold pressures. Such

relatively simple approaches do not always work well since the resolving power of SFE is rather limited. It is for this reason that SFE cleanup methods frequently use in-situ adsorbents, e.g., adding the sorbent, usually after the sample to be extracted, to impart additional selectivity over that which can be achieved by changing the variables that control SFE. Variations in this theme include "inverse" SFE and coupling matrix solid phase dispersion with supercritical fluids.

A. Fluid Density-Based Fractionation

The variation of fluid density as a function of pressure and temperature for compounds in their supercritical and near critical fluid state are available, or can be computed with a fair degree of accuracy from thermodynamic equations of state [64]. The density of a supercritical fluid goes through a substantial change at its critical point making control of its density and hence solvent power difficult to regulate in this region. Beyond the critical point, further compression yields a modest increase in density and hence solvent power; however by increasing both the extraction pressure and temperature beyond the critical temperature and pressure, an increase in a solute's solubility in a SF can be affected. Hence, with SC-CO_2, extraction of higher molecular weight or polar compounds can be accomplished under such conditions.

As noted by King [65], it is the relative solvent power of the supercritical fluid, i.e. its solubility parameter to that of the target analyte, that to a first approximation determines the extent of solubility of the analyte in the SF. Therefore, if there are significant differences in the solubility parameters of the extractable components making up the sample matrix, fractionation may be feasible. Table 4 lists some of the estimated solubility parameters of components found in sample matrices. For maximum solubility of a solute to occur in a supercritical fluid, their

respective solubility parameters must be equal, however this condition in analytical SFE is only rarely required (e.g., extraction of fat). Since the solubility parameter of the extraction fluid is directly proportional to the fluid density, it should be noted that when the solubility paramter of the solute and solvent are within 2.5 Hildebrand units (δ, cal $^{1/2}/cc^{3/2}$), some degree of mutual miscibility is assured. For SFE of trace components, even this criterion may be relaxed, and a much lower fluid density or solubility parameter will suffice for extracting the target analyte.

Solubility parameters between 0-9.0 can be attained using SC-CO_2. From the values in Table 4, it is apparent that some solubilization of fats and lipids can occur in SC-CO_2 depending on the extraction fluid density. The high solubility parameters associated with carbohydrate and proteins or amino acids suggest that these moieties will not be soluble in SC-CO_2 to any great extent. Indeed, this is what is found experimentally [66] and only at very high pressures can any recorded solubility of these substances occur. This fact makes SFE with SC-CO_2 attractive for separating many compounds from protein- and carbohydrate-sample matrices. It should be noted that the addition of cosolvents to SC-CO_2 can increase the fluid's propensity for extracting more polar analytes. For example, addition of ethanol to SC-CO_2 has been shown to extract polar lipids, such as phospholipids, that exhibit hardly any solubility in neat SC-CO_2.

In some cases, careful selection of the extraction fluid density can provide the desired degree of sample cleanup for the final analysis method. Gere and Derrico [67] have suggested that extraction fluid densities less than 0.4 g/mL will minimize the coextraction of interferring lipids during SFE. A further increase in extraction fluid density will increase the degree of lipid solubilization, and a fluid density of 0.6 g/mL will in most cases, assure the coextraction of lipids. For example, David et. al. [68] found that for the SFE of polychlorinated biphenyls from

cod liver oil, that an extraction fluid density of 0.50 g/mL for SC-CO_2 coextracted only 2 wt. % of the interferring fats, while at an extraction fluid density of 0.75 g/mL, SC-CO_2 successfully extracted all of the cod liver oil. Other examples of partial fractionation that may be useful for analysis purposes are the separation of essential oils, bitter acids, and triglycerides from hops by changing the density of SC-CO_2; or the separation of antioxidant, Irganox 1076, from a high molecular weight polyethylene film matrix [69].

Despite the examples of optimizing selectivity in SFE by changing the extraction fluid density alluded to in the previous paragraph, some mention of the special problems posed by biological and natural product matrices when conducting SFE should be noted. Although it is a mute argument as to what sample matrices are the most difficult to extract specific analytes from via SFE, it probably fair to say that the molecular complexity of many natural and biological samples poses specific problems to SFE with respect to extraction specificity. Whereas SFE of environmental matrices are simplified somewhat due to their high content of inorganic matter, the level of extractables in food and biological matrices is quite variable, despite the fact that carbohydrate and protein matter have limited solubility in SC-CO_2 under typical analytical SFE conditions. It is the high propensity of SC-CO2 to extract lipid matter from natural products however that causes much of the selectivity problem in the SFE of these materials, and has lead to the integration of cleanup techniques into the SFE schemes.

The extraction of volatiles and semivolatiles from biological matrices by SFE offers some improvement over other techniques such as headspace or purge/trap methods due to its benign nature. When performing SFE using SC-CO_2, the sample matrix in the extraction cell is in a non-oxidative environment (CO_2). When this factor is coupled with a relatively low extraction

temperature, there should be minimal degradation of the target analyte during the extraction process. SFEs using SC-CO_2 can frequently be performed under 200 bar and at temperatures slightly above the critical temperature of the extracting fluid, e.g., 35-45°C for SC-CO_2. Such SFEs conducted at lower pressures also avoids the simultaneous extraction of oil or fats which can interfere in the final analytical method, e.g., gas chromatography.

B. Use of Adsorbents with SFE

As noted previously, integration of adsorbents into the analytical SFE process, either prior to or after SFE, can often produce a sample sufficient "clean" for direct analysis. Table 5 lists typical sorbents and materials that have found use in analytical SFE. The sorbents listed generally tend to be "normal" phase column packing materials according to a liquid chromatographic or HPLC classification system. This fact is not coincidental since the elutropic strength of SC-CO2 even at high pressures is equivalent to non-polar to medium polarity liquid solvents. Sorbents such as aluminas, silicas, surface bonded silicas, diatomaceous earths, and Florisil have all been cited in the SFE literature. These sorbents can be added directly to the sample or into the extraction vessel as a segregated bed apart from the sample matrix.

As noted by Randall [70], SC-CO_2 is a weak elutropic solvent, and the analyst must be careful in selecting the proper sorbent, or tailoring its surface activity, so as to permit elution of the desired analytes. As in normal adsorption chromatography-based cleanup systems, sorbent strength can be tempered by addition of water to the sorbent before SFE to lower their surface energy. Organic-synthesized sorbents, such as adsorbent disks and synthetic resins/foams, are low surface energy sorbents, and are compatible for use with SFE, although they may be subject

to morphological change to the effect of pressure or sorption of the SF (i.e., plastization) [71].

Use of the above normal phase adsorption technique requires that several factors be assessed and controlled for the technique to work in the supercritical fluid mode. The analyte retention characteristics must be assessed as a function of the total quantity of supercritical fluid eluent passed through the sorbent bed to successfully capture the analytes. This is illustrated in Fig. 12 where the breakthrough of three organochlorine pesticides from an alumina cleanup sorbent with SC-CO_2 at 250 atm. and 50°C follows a classic sigmoidal frontal breakthrough curve. This elution pattern, expressed in terms of total expanded volume of CO_2 through the sorbent bed, was accomplished using 1.8 g of alumina in a 3.5 mL extraction cell. In this case, approximately 0.2 g of sample was initially put on top of the alumina bed.

The use of mini-chromatographic columns or cartridges in series with the SFE extraction vessell has also been reported [72] and can involve all of the retention mechanisms well known to chromatographers; namely adsorption, size exclusion and complexation. This post SFE trapping can include the use of traditional solid phase extraction (SPE) materials, or fabricated traps as has been reported for the capture of more volatile compounds. This sorbent-based chemistry can be used in several modes when coupled with SFE. Figure 13 illustrates the use of Hydromatrix as a both a sample dispersant and mild dessicant, as well as void volume filler in the extraction cell. Fig. 13 also illustrates how alumina as a sorbent can be used in its traditional format after SFE to segregate the target analytes from fat coextractives, or within the SFE cell to retain more polar target analytes that can then be eluted off the alumina bed. In the latter case, the isolated analytes can be removed from the alumina by increasing the SFE temperature and pressure, or by incorporating an organic cosolvent with SC-CO_2, or by simply emptying the cell

and using a liquid to elute the target analytes off the alumina. Such methods have been used extensively by Maxwell and coworkers [73,74] for the analysis of antibiotics in biological tissues.

An excellent example of the use of sorbent technology with analytical SFE is for the isolation of pesticides from lipid matter as shown in Fig. 14 employing the principle discussed in Fig. 12. Here, neutral alumina, initially thermally-activated, with subsequent adjustment of its final activity level via additional of water, is used to retain interfering lipid moieties while the pesticides: endrin, dieldrin, and heptachlor epoxide are eluted with high recovery with SC-CO_2 relative to conventional cleanup techniques [75]. Similar fractionations for cleanup have been reported using SFE in the selective isolation of polynuclear aromatic hydrocarbon and PCPs from environmental matrices. The addition of small quantities of cosolvent can aid in analyte recovering using the supercritical fluid cleanup (SFCU) technique, but care should be taken that breakthrough of the undesired species (lipids) does not occur.

C. Inverse SFE

Another sorbent-based method SFE invented by the author is "inverse" SFE. Here a sorbent is incorporated into the extraction cell to isolate the target analyte of interest under SFE conditions, while interfering compounds are removed by the extraction fluid. The concept is illustrated in Fig. 15 and contrasted with the normal SFCU technique. As shown in the first two sequences in Fig. 15, the addition of a adsorbent into the extraction sequence is normally utilized to yield a simplified extract containing the analytes of interest. In inverse SFE, the sorbent is added to the extraction vessel, or in-line as a separate bed, to facilitate the removal of the solutes that are

unwanted or would interare in the subsequent assay [76]. This is frequently done by using neat SC-CO_2 to remove the unwanted compounds, followed by a cosolvent/SC-CO_2 mixture or organic solvent to remove the target analytes from the sorbent bed.

Examples of inverse SFE include: the separation of lipids from leucogentian violet a coccidiostat found in poultry tissue, cleanup of extracts containing aflatoxin M_1, isolation of polymyoxin B sulfate from a pharmaceutical cream, and reduction of the interfering lipids in extracts containing cholesterol. With respect to the last case of determining cholesterol in the presence of other lipid coextractables, such a sample cleanup is of considerable importance in determining cholesterol levels in foods and biological fluids. The determination of cholesterol, can be accomplished with SC-CO_2 as reported in the literature [77-79], however the problem from an analytical perspective are the large number of coextractives that also exhibit sufficient solubilities in SC-CO_2, which are coextracted along with the desired analyte. Using "inverse" SFE [76], it has proven possible to retard the target analyte of interest, cholesterol, while removing the interfering coextractives first with neat SC-CO_2.

For example, amino-bonded silicas (from SPE cartridges) will selectively retard sterols relative to other lipid components in the presence of SC-CO_2. By using a three-fold excess of this sorbent to sample by weight in the extraction cell, interfering triglycerides were fractionated away from cholesterol at 500 atm. and 80°C. Then, by using 6 vol. % of methanol in the SC-CO_2, for the same extraction time and conditions used for the neat SC-CO_2 extraction, the cholesterol could be eluted off the sorbent, relatively free of interfering lipids (based on results from the capillary SFC analysis of the collected extract fractions).

D. Variation in Extraction Fluid Type or Composition

Although CO_2 is by far the most commonly used extraction fluid for the reasons noted above, there are several other candidates that have utility also, or niche applications. Of particular note are the fluorocarbons such as HC-134, SF_6, and fluoroform. Levy [80] has shown that SF_6 under appropriate conditions can selectively extract alkanes with respect to aromatic hydrocarbons. The hydrogen-bonding propensity of fluoroform (HCF_3) allows differential SFE to be accomplished on polar analytes, such as opium alkaloids. For example, Stahl, et al. [81] demonstrated that the alkaloid, thebaine, is preferentially solubilized over codeine and morphine by HCF_3.

Fluoroform also has a lower propensity to extract lipids, which makes it attractive for extracting analytes from fat-containing matrices. This property of HCF_3 has been exploited by King and Taylor [82] to selectively extract pesticides from poultry fat as shown in Fig. 16. More recently, Taylor, et. al. [83] have used the selective extraction properties HCF_3 to extract drugs devoid of extraneous lipid coextractives. Relative to SC-CO_2, HCF_3 under the right extraction conditions can be used rather then SC-CO_2, resulting in an extract with 100 times less fat than that obtained with SC-CO_2 under identical extraction conditions (250 atm, 50°C, 50 mL of HCF_3 or CO_2). The result from using HCF_3, as shown in Fig. 16, is that the derived extract can then be diluted and directly injected for GC/ECD analysis of the organochlorine pesticides. The resultant chromatogram in Fig. 16 allows detection of the three organochlorine pesticides from poultry adipose tissue at the 1-3 ppm level, relatively free of any interferences. This is indicative of the superior discriminating power of the HCF_3 relative to lipid coextractives.

Another approach that has been found to be effective is the use of binary supercritical fluid mixtures for fractionating target analytes and coextractives. In this case, a fluid is used that

has a considerably lower critical temperature relative to the principle solvating fluid (i.e., SC-CO_2), but both fluids are in their supercritical state. This type of binary fluid mixture has less solvating power that that possessed by the fluid having the higher critical temperature [84], but sufficient solvating power to selectively extract trace levels of target analytes from interfering substances, such as coextracted lipids. This is one of the reasons that SFE using a 70 mole % CO_2/30 mole % N_2 mixture will give extracts containing less than 5 mg of fat at 8000 psi and 60° or 80° C ,, while assuring complete recovery of pesticides at the ppm level [85].

An indication of the moderating effect of the fluid with the lower critical temperature, N_2, is shown in Table 6 where the amount of pesticide recovered along with the quantity of lipid coextracted, as a function of fluid composition at 10,000 psi and 70°C, is noted. Both pure CO_2 and a 95 mole % CO_2/5 mole % N_2 extracted 3.8 and 1.82 g of lipid respectively under the above conditions. A fluid composition of 20 mole % CO_2 /80 mole % N_2 extracts approximately zero fat, but as noted in Table 6, the pesticide recoveries are very low. An intermediate composition of carbon dioxide with nitrogen (75 mole % CO_2/25 mole % N_2 reduces the coextraced lipids to 110 mg, while yielding 70% recoveries of the target analytes. Further optimization of the binary fluid composition (8000 psi, 60°C, 70 mole % CO_2 /30 mole % N_2) permitted the recoveries of pesticides noted above with minimal coextractives.

IV. COUPLING REACTION CHEMISTRY (DERIVATIZATION) WITH SFE

A. Reactions in Supercritical Fluid Media

Analytical reactions conducted during SFE, provide the analyst with another variable to improve extraction selectivity, analyte detection of extracted analytes via derivatization, an increase in

analyte volatility, and enhanced solubility of the target analyte(s) in the extraction fluid. This can encompass quite a wide range of analyte types, ranging from lipids, pesticides, to inorganic species as shown in Table 7. Many of the cited reactions utilize well known derivatizing agents that have been used in GC and HPLC methodology, or as a reactant designed to improve the solubility of a sparingly insoluble analyte in SC-CO_2. The latter is best illustrated by the use of fluorinated "designer" ligands for enhancing the solubility of metal species in SC-CO_2 [86]. One must be cautious when using derivatizing agents as reactants in SFE, particularly with matrices that are complex, since the resultant extract may turn out to have more extracted components then obtained through a conventional SFE approach.

Reactions conducted under pressurized supercritical fluid conditions, hereafter referred to as supercritical fluid reactions (SFR), accure many of the same benefits when applying pressure to facilitate or accelerate a particular reaction chemistry. The rate constant associated with the reaction may increase (and in some cases decrease) and ultimately favor a particular reaction path way or product. Likewise, catalysts which show minimal effect under ambient conditions, may be better at facilitating a reaction under supercritical fluid conditions. Therefore, by judiciously combining SFE with SFR, one gains some of the same benefits as changing the reaction solvent in the condensed liquid state, as well as the possibility of further fractionating the resulting end products for analysis.

Exploiting a SFR for the purpose of analytical derivatization has frequently been done on an empirical basis with little optimization. This includes the choice of the best derivatizing agent as well as the optimal conditions for affecting derivatization for a particular class of analytes. A multivariate SFR/SFE optimization scheme has been used by Cela and coworkers [87] to study

the acetylation of phenolic analytes from soil samples in which nine experimental variables were optimized. In a more recent study, King and Zhang [88] examined five reagents with respect to their efficacy as derivatizing agents for carbamate pesticides. Heptafluorobutyric anhydride (HFBA) was found to be the best reagent when performing the derivatization in tandem with SC-CO_2 extraction of the target analytes, with identification of the resultant derivative confirmed by GC/MS. A standard HPLC post-column derivatization method was used to ascertain the completeness of the reaction as well as facilitate a comparison of the SFR method, with the derivatization performed in a typical organic solvent, benzene. Derivatizations run in SC-CO_2 were found to be faster and more complete than that achieved in benzene as a derivatization medium. Similarly, Chatfield et al. [89] demonstrated the general advantages of resin-mediated methylation of acidic analytes in SC-CO_2 versus acetonitrile using methyl iodide.

B. Types of Derivatizations Used in SFE

A number of researchers have devised SFE/SFR methods which have been the subject of an excellent review by Field [90]. Some of the more popular methods for derivatizing analytes in the presence of supercritical fluid media are the use ion pairing reagents [89, 91], silylation [92], formation of pentafluorobenzyl esters [93], transesterification to form methyl esters [94,95] and the novel use of chelating agents for metal analysis [96,97].

Silylation which is widely employed in GC-derivatizations has also been used in for SFR/ SFE. The major concern here is its application to complex matrices, which may yield unwanted derivatives that make the final analysis difficult as well as the presence of moisture in the sample matrix,which can negate the effect of the silylating agent. Hills and Hill [92] suggest that several

benefits may accure when using silylating agents in SFR/SFE: (1) direct reaction with the target analytes, (2) reaction of the sample matrix surface with the analyte, (3) and with the SFE-derived extract. Modification of sample matrix surface with the silylation agent, e.g., the end-capping of silanol groups, may aid in increasing the efficiency of the SFE.

Acylation agents have also been used in SFE/SFR, particularly for the extraction and subsequent analysis of phenols from environmental samples. Using acetic anhydride as the acylating agent, the extraction, derivatization, and analysis of phenols in soil samples [87] has been accomplished in high yields and recoveries. Likewise, phenolic analytes have been isolated from water samples using initially an anion ion exchange resin-impregnated disk to capture the phenolic moieties via adjustment of the solution pH. Subsequently, acetic anhydride is then added to the disk which is then rolled up and inserted into extraction vessel; followed by SC-CO_2 extraction of the phenolic acetates. Similarly Wells and coworkers [98] have collected acidic organic analytes on an anion ion exchange resin, and formed the methyl ester derivatives using methyl iodide as the methylation reagent. A wide variety of analytes can be assayed using this method, including chlorophenoxyacetic acids, pentachlorophenol; succinic, fumaric and citric acids, and albendazole.

Esterification reactions of organic solutes in SC-CO_2 have been extensively studied, not only for analytical purposes, but for process reaction potential [99]. Greibrokk and coworkers [100] were one of the earliest groups to demonstrate the possibilities of an enzymatic-initiated transesterification, both in the off-line and on-line modes of SFE, for the formation of the butyl esters of vegetable oils. Other investigators have used methanol/HCl on a cross-linked polymeric resin to methylate fatty acids or acidic alumina with methanol to form the fatty acid methyl esters

of the free acids or from vegetable oils. For example, King et al. [101] demonstrated that both off-line and on-line that the fatty acid methyl esters (FAMEs) of common vegetable oils could be formed by using methanol and a cosolvent (reactant) in conjunction with an alumina cleanup column, yielding FAME profiles equivalent to those found by GC analysis. Likewise, the methyl esters of natural pyrethrins were formed by Wenclawiak et al. [102] during SFE, using acidic alumina with methanol at a very high temperature, 270°C, at 40 MPa.

A more environmentally benign method of performing transesterifications is to employ an enzymatic catalyst, such as a lipase, for FAME formation. This approach has been exploited extensively in the author's laboratory based on the excellent qualitative and quantitative results achieved with lipid standards [103], oils and fats [104, 105], and for nutritional labeling analysis [106]. Using Novozyme 435 as the active lipase in the presence of SC-CO_2, facile extractions and FAME formation can be achieved at pressures ranging from 10-30 MPa and temperatures of 40-70°C. Examples of using this SFE/SFR approach as an alternative to organic solvent-based methods will be documented in the next section.

Finally, recent developments in SFE utilizing SFR and special derivatization reagents, have permitted the analysis of metals and radioactive species, such as lanthanides and actinides. Space does not permit a detailed discussion of this new aspect of SFE/SFR, but the publication of Lin et al. [86] provides a nice summary of the various chelating and derivatization reagents that have been found suitable for this purpose. A rationalization on the choice of ligands for SFE of toxic heavy metals (Cu^{+2}, Pb^{+2}, Cd^{+2}, and Zn^{+2}) from environmental matrices has been made on the basis of solubility parameter theory [107], and a model for the SFE of uranium with SC-CO_2 offered by Clifford et al. [108]. Such developments indicate that this new application of SFRs is

becoming well characterized, and along with the SFE of more volatile elemental species, such as mercury [109] and sulfur [110], may allow a nearly total analysis of the periodic table.

C. Utilization of Catalysts with SFR

Catalysts are used in the presence of SFs for many of the same reasons they are employed in high pressure catalytically-initiated reactions, i.e., to accelerate the desired reaction. Their efficicay in the presence of SFs must be evaluated since the dense fluid phase can compete for the available surface area or catalytically-active sites [111]. Field [90] has provided some examples of typical catalyst-derivatization reagent pairings which have been used during SFE/SFR. Inorganic-type catalysts as well as enzymes can be reused or regenerated in the presence of SF media, an option that is particularly attractive to the analyst. Even without the possibility of reuse, an expensive catalyst may be justified in terms of the overall time and expense associated with the sample preparation method.

The mechanism of catalytic reactions the presence of SFs may be of secondary interest for analytical purposes, but the reader can consult the extensive reference by Jessop and Leitner [112] for further reading on the topic. However, catalysts can serve multiple purposes during SFE; for example the use of tetraalkylammonium salts during SFE/SFR have been implicated for the following: (1) as a phase transfer agent effecting the solubility in the SFE step, (2) as a catalyst for a alkylation reaction, (3) as a post-SFE derivatization reagent during analysis (for example in a GC injection port), and (4) a reagent yielding a volatile reaction by-product. Controversy has arisen for the exact mechanism in which the tetraalkylammonium salts faciltate derivatization in conjunction with SFE [90], but useful methylations are the end result.

Similarly, the mode of catalytic action maybe adjusted when using enzymes in SFE/SFR. As shown in Fig. 17, alcoholysis or tranesterifications of lipids containing a ester group can be facilitated using a lipase under relatively anhydrous conditions, while hydrolysis is favored using the same lipase at higher water concentrations. Likewise, in the presence of SC-CO_2, the same conditions can be used in the extraction cell to prepare a sample for subsequent analysis. As noted in the last section, triglycerides can be made to undergo methanolysis [104,105], for the SFE and formation of FAMEs from fats/oils; however Turner, et al. [113] have applied the same lipase for hydrolyzing fat soluble vitamins in the presence of SC-CO_2.

Not all catalytic agents are equally active under the conditions of SFE. King et al. [114] demonstrated that the percent conversion for the reaction of methanol with oleic acid to form the methyl ester in a recirculating reactor for 2 hrs at 70°C and 20.5 MPa, varied with catalyst type. Conversion occurred in the order: supported p-toluene sulfonic acid > Novozyme 435 > acidic alumina > cation exchange resin (H^+ form) > titanium silicate; and only the first two catalysts proved practical for methyl ester formation.

Screening of catalysts for SFE/SFR can be accomplished with the aid of automated SFE instrumentation as described in Section IIB. In this case, the SFE/SFR technique is "inversed" to permit the evaluation of enzymatic activity under supercritical conditions, and hence the efficacy of different enzymes for a specific task. This can be done quite conveniently and rapidly using automated analytical SFE instrumentation in a combinatorial mode [115]. Table 8 tabulates the results of surveying various lipases for their ability to facilitate methanolysis of the following lipid substrates in SC-CO_2 at 17.2 MPa and 50°C over 80 min., for a triglyceride-containing shortening, cholesteryl stearate, and phosphatidylcholine. Note that Novozyme 435 assures

methanolysis of all of the above lipid moieties under the stated conditions, while Lipase G, Lipozyme IM, and Chirazyme L-1 were slightly inferior and substrate dependent. It should be noted that eight other lipases in this study failed to show sufficient catalytic activity under the above conditions, and that there was no correspondance to their ability to hydrolyze the same substrates in an aqueous buffer solution.

Turner et al. [116] ran preliminary tests on the hydrolytic activity of lipases in a SC-CO_2 for the enzymatic hydrolysis of vitamin A, retinyl palmitate, at 25.3 MPa and 60°C over 25 min using lipases derived *Candida antarcticaB*, *Pseudomonas cepacia*, and *Rhizomucor miehei*. It was found that at a water activity level of 0.43 (a_w = 0 .43), that the lipase derived from *Candida antarctica* was best for the hydrolysis of Vitamin A in the presence of SC-CO_2. Three additional lipases and one esterase that were also evaluated did not show sufficient hydrolytic activity to warrant further investigation. Thus activity levels and reaction efficiencies may change with the solvent media, a_w, and the type of reaction for a specific enzyme.

V. APPLICATIONS OF CRITICAL FLUIDS FOR SAMPLE PREPARATION

There are many applications that have been documented using SFs for sample preparation. Key reference texts which enumerate many of these applications are cited in the introductory section. In this section specific applications have been chosen to illustrate the value of utilizing SFs in terms of simplifying sample preparation, reducing the use of chemicals, as well as savings in terms of cost and labor. Most of the examples that have been selected are from the author's and his coworkers research on method development for regulatory agency use in the United States. Despite this focus, the examples illustrate both the application and potential of SFE and SFs for

sample preparation.

Table 9 lists areas of application in which analytical SFE has been applied successfully. Within each generic class of compounds in Table 9, there are certain compounds or subclasses that have not been extracted successfully using SC-CO_2, such as the beta-lactam drugs. The results obtained with SFE are also somewhat matrix dependent, therefore certain pesticides that are extracted successfully from foods maybe more problematic, or require a change in conditions, for removal from soil matrices. However this is also true when using other sample preparation methods. Overall pesticides as a compound class extract well using SC-CO_2 or SC-CO_2/modifier mixtures.

Analytical SFE has also experienced success when applied for the analysis of drugs in both foods, biological matrices, and pharmaceutical preparations. In this field of application, it is not unusual to employ a small quantity of cosolvent dissolved in SC-CO_2 to facilitate extraction of the drug from the sample matrix. Early success using SFE was recorded in the environmental analysis field, particularly in the extraction of organochlorine pesticides and dioxins, polynuclear aromatic hydrocarbons, and total petroleum hydrocarbons (TPH) resulted in the issuance of several official EPA methods. One of these, the TPH method utilizes SC-CO_2 as a solvent replacement for a fluorocarbon used previously in the method.

A. Analysis of Trace Components

Off-line SFE has enjoyed considerable success when applied to the analysis of trace components in foods; especially as a replacement extraction technique for traditional methods that use large quantities of organic solvents. Applicable trace components that can be extracted and cleaned up

using SFs include pesticides, antibiotics, natural toxins, and substances that are indicative of food adulteration. Quantitative extractions have been achieved down to the sub parts-per-billion (ppb) level [117,118] and several standard methods using SC-CO_2 have been developed and are now in routine use by regulatory agencies, particularly those involving pesticide residue analysis.

A wide range of pesticides can be analyzed using SFE, although polar pesticides may require the use of a cosolvent solvent. Initial SFE studies involved the removal of pesticides from both hydrophillic and fat-containing samples, in which control of the amount of coextracted water or fat was desired [117,118]. For example, Hopper and King [117] demonstrated that the addition of Hydromatrix to a sample not only allowed for adequate matrix dispersion, but the SFE of high-water containing samples (e.g., lettuce containing 95% water). By contrast , the use of Hydromatrix also allowed the SFE of viscous, high fat samples such as peanut butter which contained 52% fat and 2% moisture. The average recovery for 30 different types of pesticides was over 85% at incurred concentration levels ranging from 0.0005 to 2 parts-per-million (ppm).

Similar results were obtained by Snyder et al. [119] for the SFE of incurred organochlorine pesticides from various types of poultry tissues (peritoneal fat, breast, leg/thigh, liver). In this study, the SFE recovery results for the pesticides from liver tissue were found to be higher than those obtained by conventional solvent extraction. This result was ascribed to ability of the SF to better penetrate the sample matrix (liver) and to extract the target pesticides from this particular tissue matrix. It should be noted that this study stands in marked contrast to those which report the SFE of pesticides from "model" matrices, such as Celite [120], since it is important to verify that SFE can be successfully applied to an actual target matrix, preferable containing incurred residues whenever possible.

Cleanup of the SF extract can be accomplished on line, as noted in Section IIIB, however it more common to decompress the pesticide-laden extract onto a sorbent filled column/cartridge and use conventional liquid-based cleanup methodology. For example, Jones [121] extracted 8 fortified pesticides in wool wax using SC-CO_2 obtaining recoveries between 85-108% using collection in toluene. The resultant extracts were then cleaned up using a silica column. Hopper [122] on the other hand, applied SFE and cleanup on organochlorine and -phosphorus pesticides at 4000 psi and 95°C by decompressing the CO_2 directly onto a C_1 silica-based column, and then conventional cleanup methodology before final analysis. Pensabene et al. [123] applied SFE for the removal of triazine-type herbicides (both incurred and fortified) from egg matrices by decompressing onto a off-line mini Florisil column. Subsequent extract cleanup required only 8 mL of solvent. This approach was also used by King et al. [124] to extract grain samples which contained a multi-residue mixture of both spiked and incurred pesticides using the home-built apparatus shown in Figure 18. In this case a Florisil trap was inserted between a micrometering valve (MV) and the gas totalizer (GT) to permit isolation of pesticide residues on the sorbent column. Table 10 tabulates a portion of the results from this study for the SFE of 8 fortified pesticide residues at the 0.1 ppm level in wheat; extracted at 345 bar for three temperatures: 40, 60 and 80°C. By most standards, the listed recoveries on duplicate samples are certainly acceptable at all three extraction temperatures, however the results are optimal at 60°C. Note that even an incurred residue, methyl chloropyrifos, was found consistantly at the 0.04 ppm level.

SFE can also be used to advantage for the trace analysis of marker compounds which are indicative of food adulteration. Snyder et al. [125] used gas chromatography coupled with mass spectrometry to detect naphthalene and other aromatic hydrocarbons in meat matrices that had

resolution gas chromatographic analysis of the methyl esters of the constituent fatty acids which comprise the fat moieties in the food matrix. Such a procedure presented a challenge to develop an alternative SFE-based method.

To establish a baseline, a method was developed whereby all steps that were inclusive in the NLEA solvent-based-extraction protocol were utilized in a procedure incorporating SFE with SC-CO_2, rather then the specified liquid solvent [128]. This off-line SFE method utilized a sorbent disk to entrap the resultant lipid precipitate from the meat sample after acidic hydrolysis of the meat sample via filtration. The disk containing the fat precipitate was subsequently placed inside an extraction cell and the fat removed by SFE using CO_2. Trials on two different commercial SFE units indicated that the technique was not instrument dependent. Further, comparison of the results from the SFE procedure with those obtained via the traditional solvent-based protocol were equivalent for nine different meat matrices representing different levels of fat and types of meat. This procedure however was exacting and difficult to reproduce in the hands of a unskilled analyst.

Utilizing the previously described lipase-based method for transesterifying lipids, King and coworkers [105,106] developed alternative methods for producing the FAMES required for the NLEA-based method for determining fat content. Both off-line and on-line modes of SFE/ SFR (supercritical fluid reaction) were developed utilizing lipase-catalyzed transesterification, that could be employed on small representative samples. Extraction/reaction conditions of 12.2 MPa and 50°C using Novozyme 435 were found to yield both reproducible and quantitative FAME distributions on different types of dehydrated meat matrices of varying fat content (15-40% by wt.). Comparison of the fat content determined by enzymatic formation of FAMEs using

extract has been accomplished, since ECD, FPD, etc. are insensitive to contaminents that can foul the GC column. Nevertheless, applying SFE with integrated extract cleanup may reduce the need to refurbish the injection end of capillary GC columns by reducing the non-volatile solutes that are injected onto the column (see Section VA).

As noted in the introduction, there have been a number of applications of analytical SFE for the analysis of lipid or lipid-derived volatile and semi-volatile compounds. This is in part due to the relative benign extraction conditions used during SFE which minimize the formation of thermal or oxidative by-products. In addition, by applying SFE, the analyst can extract higher molecular weight, semi- volatile compounds that are not readily extracted by other techniques, thereby providing additional information. For example, Snyder and King [129] contrasted the volatile/semi-volatile profiles obtained from a thermal desorption technique with those obtained by desorption using SFE. They found two important differences between the two techniques: (1) using SFE for desorption, yielded higher molecular compounds normally not accessible via thermal desorption which could be used to further characterize the oxidative state of a seed oil, and (2) there was an absence of low molecular weight degradation products in the SFE desorption profile. The latter observation suggests that the conventional thermal-based desorption technique produced artifacts from the technique, i.e., headspace analyis-purge and trap, that were not in the original sample. The absence or limited quantity of volatiles having a carbon number less than C_6 at equivalent extraction (desorption) temperatures strongly supported this conclusion. Another advantage in using SFE for volatiles analysis is that a larger quantity of volatiles and semi-volatiles can be extracted more rapidly then when using competitive techniques.

Analytical SFE of lipid-derived volatiles/semi-volatiles has been used to study additional problems. Morello [130] applied analytical SFE to the characterization of aroma volatiles in extruded oat cereals, and noted the increased intensity of hexanal, 2,4-decadienal, and pyrazine in the SFE extract.. Seitz et al. [131] characterized the volatiles obtained from whole and ground grain samples using two methods: SFE and a helium purge technique, characterizing both extracts by off-line GC-MS/IR (gas chromatography-mass spectrometry/infrared spectroscopy). The extraction of volatiles from the ground grain by SFE was optimal at extraction pressures less than 14 MPa in the temperature range from 50 - 90°C, however the helium purge method yielded a greater quantity of volatiles for analysis. Moreover extraction using SFE proved optimal with respect to aldehydes, for 2,3-butanediols, and halogenated anisoles.

An interesting example of the application of the SFE/SFR (supercritical fluid reaction) technique prior to gas or supercritical fluid chromatography analysis, is the analysis of the fatty acid content of an industrial by-product called soapstock [132]. This rapid method consisted of mixing the sample with Hydromatrix, quickly freeze-drying the mixture, and then extracting and derivatizing the extract simultaneously using the SFE/SFR technique. The benefits of using this technique are illustrated in Fig. 20, where the SFE/SFR technique is contrasted with the AOCS (American Oil Chemical Society) Official Method G3-53 [133]. Note that the AOCS method consists of many manual steps, takes 5-8 hours to perform depending on the analyst, and requires over half a liter of organic solvent. However, the SFE-based method takes only 3 hours and utilizes less than 2 mL of solvent! An alternative method, which is quite rapid but gives slightly lower results than either the AOCS or SFE/SFR method, uses capillary SFC for the analysis of the soapstock sample. This method takes only 45 minutes and uses only 8 mL of solvent. Such

rapid methods find application in industry, thereby permitting the quick diagnosis of problematic shipments of soapstock.

D. SFE with HPLC or SFC

The use of off-line SFE in conjunction with either HPLC or SFC has been reported many times in the literature. The coupling of SFC with off-line SFE is a logical extension of the use of SC-CO_2, has been noted by King and Snyder [134], since extracts obtained using SFE with CO_2 should be amenable to analytical chromatography using the same SF. The ability to use pressure or density programing in SFC for the resolution or removal of unwanted higher molecular solutes components extracted during the SFE step is another key advantage of using off-line SFE/SFC as cited by King [135]. HPLC like GC offers the opportunity to use selective detectors such as UV, photodiode-array UV, or fluorescence, which can mask responses from unwanted and coeluting solutes in the final chromatographic assay. Non-specific modes of detection, such as the FID in SFC and evaporative light scattering detector (ELSD) in both HPLC and SFC have found use in characterizing SF-derived extracts.

The studies of Maxwell and coworkers in which off-line SFE was applied for the analysis of drugs are excellent examples of coupling SFE with HPLC. For example, sulfonamides were isolated from chicken tissure using SC-CO_2 at 10,000 psi and 40-60°C, using an in-line trap of alumina to trap the target analytes [136, 137]. The analytes were then eluted off the sorbent with the HPLC eluent allowing a detection sensitivity of 100 ppb to be realized. Similarly, HPLC with photodiode array detection was used to quantify zoalene and its metabolites in chicken liver [138]. It was found that excessive dehydration of the liver tissue prior to SFE was deleterious to

the recovery of zoalene, however the addition of a small quantity of water to the liver tissue/Na_2SO_4 mixture in the extraction cell permitted 90% recoveries of zoalene and one of its metabolites. Another method developed by Parks et al. [139] using both HPLC-UV or GC-MS employing the hexafluorobutyric anhydride (HFBA) derivative, allowed the determination of melengesterol acetate in bovine fat tissue down to the 25 ppb level with over +99 % recovery of the analyte. As shown in Table 12, the method developed for melengesterol acetate which uses SFE, results in a considerable savings in solvent use. The Food Safety & Inspection Service (FSIS), Food and Drug Administration (FDA), and Association of Official Analytical Chemists (AOAC) methods all require between 1.7-2.2 L of organic solvents; many of the solvents corresponding to those that are carcinogenic such as chloroform, benzene, and methylene chloride ($MeCl_2$). In addition, both the recoveries and precision of the SFE method for melengesterol acetate are superior to those obtained with the solvent-based regulatory agency methods. Addition information on these SFE methods that employ in-line sorbent trap as discussed in Section IIIB and Figure 13 can be found in the reviews by Stolker et al. [140] and Maxwell and Morrison [141].

Multiple HPLC detectors and SF-based sequences can be coupled to advantage in the development of methods. Recently, researchers [142,143] at Lund University in Sweden have used an integrated enzyme-initiated reaction to hydrolyze fat soluble vitamins in-situ during the SFE of vitamins from a variety of food matrices. Using Novozyme 435 at 60°C and 25.9 MPa (a SC-CO_2 density of 0.8 g/mL), and 5 % vol. of ethanol, they successfully extracted foods containing vitamins A and E. They found that vitamin A could be readily hydrolyzed under SFE conditions to retinol which could then be determined by HPLC both using ultraviolet and/or

Using the above two-step SFE fractionation method, on a soybean oil contained 0.11% beta-sitosterol, 0.06% stigmasterol and 0.04% campesterol; indicated that the initial SFE step removed 95% of the triglycerides. Upon application of the second SFE step the concentration of sterols increased from 0.21 % in the initial extract to 25 %. Similar results were also achieved on other vegetable oils as shown in Table 13. These results indicate that the two-step fractionation method can produce a substantial enrichment of sterols from seed oils for analytical detection. An extension of this method using four discrete SFE steps and methyl t-butyl ether as a cosolvent has been reported by Snyder et al. [144].

Extraction of polar analytes from biological matrices by SFE presents some of the same problems as SFE of analytes from environmental matrices. This is due to the fact that some analytes may be sparingly soluble in SC-CO_2 and/or be tightly bound to the sample matrix so as to require the use of a cosolvent along with CO_2. The choice of cosolvent and its quantity along with the extraction conditions can require many independent experiments to optimize the final SFE method. The use of automated SFE instrumentation along with a combinatorial evaluation approach can greatly accelerate the development of a final extraction method [145].

An illustrative case is the extraction of of the mycotoxin, aflatoxin B_1 from yellow corn which requires the use of a binary modifier to obtain successful recoveries. Extraction with neat SC-CO_2 proved unsuccessful, even at pressures up to 1,034 bar and high temperatures (80°C). Static addition of small aliquots of several modifiers also proved insufficient relative to dynamic addition of the cosolvents. A 2:1 acetonitrile/methanol modifier mixture [146] was then tested using different extraction temperatures, pressures and percent modifier a shown in Table 14. As indicated, 15% of the binary modifer at 5000 psi (345 bar) and 80°C proved sufficient to give

recoveries equivalent to those obtained via solvent extraction. HPLC with fluorescence detection was used to determine the concentration of aflatoxin B_1 in the extracts which were derivatized with trifluoroacetic acid (TFA) to convert aflatoxin B_1 to B_{2a} for enhanced detection.

Will such conditions suffice for the same or similar mycotoxins in different matrices and at different levels of contamination? Additional research has shown that much lower levels of aflatoxin in yellow corn (15ppb versus 600 ppb in the above example), that aflatoxin B_1 is only recovered at a 60% level using the above optimal conditions. This low recovery may reflect the difficulty in extracting the lower level of trace analyte from the yellow corn matrix. Similar results have also been recorded for the recovery of aflatoxin B_1 from white corn, indicating that the method does not have universal applicability to a variety of sample matrices. Using the above approach, extractions were attempted of the more polar aflatoxin B_1 metabolite, aflatoxin M_1, from beef liver at a 0.3 ppb level. Liver is a notoriously difficult matrix to extract analytes from as noted previously, and the use of cosolvents frequently requires the need for extract cleanup after completion of SFE. Nevertheless a reasonably clean SF-extract can be achieved by conducting the extraction at 552 bar and 80°C, using only 3.3 vol. % of 2:1 acetonitrile/methanol modifier, yielding a 86% recovery [146]. Although such method development is often arduous, as it is even when using conventional liquid extraction, it demonstrates the experimental flexibility that makes analytical SFE an attractive technique.

E. SFE Integrated with Selected Chromatographic/Spectroscopic Techniques (IR, MS)

SFE has been used in conjunction with an assortment of spectroscopic techniques, often in combination with a chromatographic separation method, to allow a virtual "alphabet soap" of

possibilities as noted in Table 15 [147]. Many of the off-line SFE-based methods are similar to those used in integrated on-line SFE systems, perhaps the most popular being SFE-GC-MS, SFE-SFC-MS, and SFE-IR. As commented on the introductory section, several references review the use of on-line SFE with various spectroscopic instrumentation, Ramsey's [11] perhaps being the most current. To illustrate the potentially wide range of applications that can be investigated using SFE and spectroscopic detection, several select studies are described below.

Liescheski [148,149] has coupled infrared spectroscopy on-line with SFE to determine the iodine number of edible oils as well as the trans fatty acid content of vegetable oils. In the former case, it was found that the symmetric CH_2 stretching frequency could be linearly correlated with the iodine number. Direct transfer of the dissolved lipids to an on-line IR cell from an Isco SFX 2-10 unit was used in the reported experiments. Liescheski has also used the SFE-IR tandem technique to determine the total lipid content of milled rice flour.

A particularly novel application of analytical SFE related to lipid technology is its use to detect irradiated foodstuffs. In a landmark study, Lembke et al. [150] used SFE and GC-MSD (mass selective detector) to characterize the hydrocarbon patterns and appearance of cyclic ketones that were characteristic of foods exposed to irradiation. By using a low extraction fluid density, 0. 25 g/mL, the marker hydrocarbons could be readily extracted avoiding the SFE of higher molecular weight fatty acid moieties. Among the irradiated foods extracted were pork meat, duck breast, pastachio nuts, and chicken soap.

Both Tewfik et al. [151] and Stewart et al. [152] used analytical SFE to extract the 2-alkylcyclobutanone moieties from irradiated foods. Exposure of foods to irradiation yields straight chain hydrocarbons that are one carbon number less than the parent fatty acid, i.e., odd

numbered fatty acids that are reliable markers for food exposure to irradiation. The 2-alkyl cyclobutanones arise from fatty acids of the same carbon number and have the alkyl group in a ring position, therefore fatty acids such as palmitic, stearic, oleic, and linoleic can yield trace levels of the alkylcyclobutanones. As shown in Table 16 [152], extraction using SFE shows an increasing concentration of alkylcyclobutanones with irradiation dosage for three commodity food items. It should be noted that the analytical method using SFE and GC/MS for cyclobutanone detection takes approximately six hours to perform, while the standard method takes two days to arrive at the same results.

Multiple couplings or uses of SFE can also put to advantage in analyzing ingredients in complex food samples. Huang et al. [153] identified and quantified the fat-reducing ingredient, Salatrim, in cookie, bonbons, and ice cream using SFE in combination with particle beam LC-MS and HPLC using an evaporative light scattering detector (ELSD). The fat content of the above matrices was also determined using SFE, while the particle beam LC-MS system using the ammonia chemical ionization mode, was used off-line to determine the triacylglycerol that are characteristic of Salatrim. Quantification on the SFE samples was done on the HPLC/ELSD system. This is a nice example of the compatibility of off-line SFE with different analytical methods.

VI. STATUS OF THE TECHNIQUE - CONCLUSIONS

In conclusion it would appear that overall SFE-based methods have a promising future in food analysis, particularly for sample preparation involving the analysis of fats, pesticides, specific drug moieties, trace toxicants, and food adulteration. It has been demonstrated that analytical

SFE can be reproducibly used over a wide range of analyte concentrations, ranging from 1-50 weight percent down to ppb levels. Slowly several collaborated or peer verified methods have evolved involving the determination of fat/oil levels in food and natural product matrices, for the determination of pesticide residues, and a recent European Union-sponsored study involving the determination of fat soluble vitamins in foodstuffs. However, more collaborative and/or peer validated methods will be needed in the future to substantiate SFE as a sample preparation tool. Currently several food companies in the United States utilize multiple SFE units for routine fat determinations in a production plant environment. In one specific case, the SFE results are used to calibrate an on-line infrared analyzer used in food production lines.

Additional future trends are nicely sumarized by Luque de Castro and Jimenez-Carmona [154]. These include the use of pressurized fluids, such as subcritical water as an alternative to SC-CO_2 as an environmentally- and worker-friendly solvent. Recently Curren and King [155] have also demonstrated the utility of pressurized water or water/ethanol mxitures for the extraction and sample preparation of pesticides from fortified meat tissues. Another area of application for analytical SFE is in the field of nutraceuticals, where it is a logical extension of SC-CO_2-based processes that are used for the extraction and enrichment of key nutraceutical ingredients [156]. Therefore it becomes possible using some of the specific instrumentation discussed in Section IIB, to combinatorially-optimize analytical or process SFE, as would be required for the complex products that are used in nutraceutical product development.

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VIII. APPENDIX I

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Table 1 Desired Features in Analytical SFE

Pressure, Temperature and Flow Rate Ranges
Sample Size Range
Variety of Collection Options
Size and Portability of Instrument
Automation
Cosolvent Capability
Ability to Interface with Other Instruments
Delivery System for Carbon Dioxide

Table 2 Extraction of Aflatoxin B_1 from Corn

Method (N = 5)	Average Recovery	Relative Standard Deviation
CB Method**	441.4 ppb	3.2
SFE Method*	519.6 ppb	6.2
Method 1**	549.2 ppb	3.5
Method 1*	515.1 ppb	10.5

* 3.0 gram samples

** 50.0 gram samples

Table 3 Options for Integrating Sample Cleanup with SFE

Fluid Density-Based Fractonation
Supercritical Fluid Adsorption Chromatography
Integration of Selective Adsorbents
Alternative Fluids to Carbon Dioxide
On-Line SFE/Chromatography Methods
Inverse SFE
SF-Modified Size Exclusion Chromatography (SEC)
Use of Binary Gas Mixtures

Table 4 Characteristic Solubility Parameters (δ) and Their Relationship

For Maximum Solubility: $\delta_{solute} = \delta_{SF}$

For Miscibility: $(\delta_{solute} - \delta_{SF})$

Characteristic Solubility Parameters (δ)

Compound	δ	**Compound**	δ
$SC\text{-}CO_2$	0.0-9.0	Proteins/Amino Acids:	
Fats/Lipids	8.5 - 10.0	Valine	11.0
Water	23.5	Histidine	15.3
Carbohydrates:		Tryptophan	13.1
Glucose (Cal'c)	18.9	Glycine (Cal'c)	13.0
Sucrose in H_2O	22.0	BSA	11.7 - 14.7
		Blood Serum	21.7

BSA = Bovine Serum Albumin

Table 5 Sorbents Used for Fractionationation of Extract

Aluminas	Silica Gel
Silicas	Florisil
Celite	Hydromatrix
Silyated Silicas	Synthetic Resins

Table 6 Pesticide Recoveries and Lipid Extracted from Poultry fat as a Function of Fluid Composition

	Fluid Composition (mole %)			
	Pure CO_2	95% CO_2/5% N_2	75% CO_2/25% N_2	20% CO_2/ 80% N_2
Lipid (mg)	3800	1820	110	0
	Pesticide Recovery %			
Heptachlor*	100	100	70	6
Dieldrin	100	100	70	11
Endrin	100	100	65	9

* As epoxide

Table 7 Reactions and Derivatization Applied in Analytical SFE

Alkylating Agents/BF3 - Acidic Herbicides
Ion-Pairing Agents/TMPA - Ionic Surfactants
Pentafluorobenzyl Esters/TEA - Phenols, etc.
Silylation Reagents - Matrix Derivatization
5% HCl/Methanol/XAD-4 Resin - Fatty Acids
Trimethylphenylammonium Hydroxide - Fatty Acids
Acetic Anhydride/AG-1-X8 Resin - Phenols
Esterification - Tocopherols
Lipase/Alcohol Transesterification - Fatty Acids
Alumina/Alcohol - Fatty Acids
Ligand Reactions - Assorted Metals/Inorganics

Table 8 Lipase-Catalyzed Methanolysis for SFE/SFR Conversion of Lipids (%)

Lipase	Shortening	$C_{18}CE^{*}$	PC**
Lipase PS30[a]	2	10	1
Lipase L[a]	4	1	N.R.
Lipase Ay[a]	5	1	N.R.
Lipase MAP10[a]	56	31	22
Lipase G[a]	90	100	48
Pseudomonas cepacia Lipase[b]	81	45	80
Novozyme 435[c]	100	98	99
Lipase from *C. Antarctica A.*[c]	1	N.R.	N.R.
Chirazyme L-1[c]	100	98	90
Chirazyme E-1[c]	6	2	1
Lipozyme Im[c,d]	99	96	60

$C_{18}CE$ = cholesteryl stearate

PC = Phosphatidylchloine

[a]Immobilized on Accurel.

[b](Sol-gel) Reaction products included 15% monoglycerides and 19% diglycerides.

[c]Carrier-fixed (not specified by manufacturer).

[d]Reaction products included 16% monoglycerides

Table 9 Applications of Analytical SFE

Pesticides
Petroleum Products
Environmental Samples
Fat and Lipid Analysis
Drugs and Antibiotics
Polymer Oligomers/Additives
Metal Analysis
Volatiles and Flavors

Table 10 Percent Pesticide Recoveries from Wheat at 0.3 ppm Fortification Level

Conditions: 345 bar, 100 L-CO2 (expanded)

	40 C		60 C		80 C	
	10A*	10B	11A	11B	12A	12B
Dimethoate	88	87	82	101	77	84
Methyl Parathion	89	89	92	103	91	93
Pirimiphos Methyl	96	95	101	108	99	100
Chlorpyrifos	97	97	105	113	99	101
Malathion	93	95	102	109	96	97
Dieldrin	95	91	104	104	93	91
Methoxychlor	94	94	85	107	97	103
Carbofuran	89	97	97	98	92	95
			Incurred Residue Results (ppm)			
Methyl Chlorpyrifos	0.039	0.038	0.042	0.043	0.044	0.041

Table 11 Concentration of Naphthalene (ppb)[a] in Meat Samples by SFE/GC/MS

Type of Meat	Control (RSD)[b]	Fire-Exposed (RSD)[b]
Beef roast	0	10.7 (8.5)
Boneless beef	LOQ	3.5 (4.1)
Corn beef	1.7 (14.2)	14.6 (3.1)
Ham	2.5 (12.8)	21.3 (4.8)
Smoked chicken	11.7 (5.3)	50.8 (0.6)
Turkey breast	LOQ	4.3 (4.0)
Boneless turkey	0	6.2 (0.8)

[a]Concentration determined using naphthalene-d^8 as the internal standard.

[b]RSD, relative standard deviation was determined from three extractions.

[c]LOQ, limit of quantitation in 1 ppb

Table 12 Comparison of Organic Solvent Consumption for Recoveries of Melengesterol Acetate

Method	Recoveries (% +/- RSD)	Solvent Used (L)	Solvents
FSIS	96.7	>1.9	Hexane Acetone Acetonitrile
FDA	74.4 +/-8.0	>2.2	Hexane Methanol Ethanol Chlorofomr Diethyl Ether Benzene $MeCl_2$
AOAC	93.0 +/- 7.5	>1.7	Hexane Acetone Benzene Acetonitrile
SFE	98.4 +/- 4.5	0.012	Methanol**table**

Table 13 Concentration of Sterols in Seed Oils by Supercritical Fractionation

Seed Oil	Initial Amount	Amount After SFE
Corn Oil	0.2 %	21%
Canola Oil	0.7 %	33%
Cottonseed Oil	0.3 %	28%
Soybean Oil (hexane)	0.2 %	18%
Soybean Oil (SFE)	0.2 %	25%

Table 14 Screening for Optimal Conditions for the SFE of Aflatoxin B_1 from Corn Sample

Pressure (bar)	Temp. (C)	% modifier*(vol)	Vol of CO2 (mL)	Recovery(ppb)
345	80	5	100	476
345	80	10	100	274
345	80	15	100	595
345	80	20	100	342
517	40	5	100	446
517	40	10	100	502
517	40	15	100	459
517	40	20	100	282
CB Method**				449
No Cleanup***				595

* ACN/MeOH (2:1), ** silica column cleanup of $HCCl_3$ extract, ***No silica column cleanup

Table 15 The "Alphabet Soap" of Hyphenated Supercritical Fluid Techniques

SFE - GC	SFE-HPLC	SFE-GPC
SFE-IR	SFE-FTIR	SFE-GC-MS
SFE-SFC-MS	SFE-GC-AED	SPE-SFE-GC
SFE-SFC-FTIR-MS	SFE-GC-IR-MS	SFC-UV-FTIR-MS
SFE-IMS	SFE-TLC	SFE-UV

GPC = gel permeation chromatography

AED = atomic emission detector

SPE = solid phase extraction

UV = ultraviolet spectroscopy

IMS = ion mobility spectroscopy

TLC = thin-layer chromatography

Table 16 Concentrations of 2-Dodecylcyclobutanone (2-DCB) and 2-Tetradecylcyclobutanone (2-TCB) Isolated by SFE from Irradiated Foods

Foodstuff	Irradiation Dose	2-DCB*	2-TCB*
Chicken Meat	0.5	0.02	0.01
	2.5	0.10	0.03
	5.0	0.14	0.05
Liquid Whole Egg	0.5	0.06	0.03
	2.5	0.57	0.36
	5.0	1.23	0.57
Ground Beef	0.5	0.06	0.06
	2.5	0.35	0.36
	5.0	0.63	0.57

* Concentrations in micrograms/10 gram of sample

FIGURE LEGENDS

Fig. 1 Phase diagram of carbon dioxide.

Fig. 2 Solubility of naphthalene in SC-CO_2 under conditions corresponding to those used during extraction and separation (sample collection).

Fig. 3 The analytical SFE triangle.

Fig. 4 SFE of ham sample as a function of extraction time

Fig. 5 Generic laboratory SFE unit. A = CO_2 cylinder; TP = cylinder pressure gauge; CV = check valve; F = filter; C = air-driven gas booster compressor; RV = relief valve; SV = on/off switching valve; PG = pressure gauge; HC = equilibration coil; Tc = thermocouple; MV = micrometering valve; FM = flow meter; GT = gas totalizer.

Fig. 6 Simultaneous, parallel multi-sample SFE unit.

Fig. 7 Effect of sample size on extraction time and precision.

Fig. 8 Solubility of soybean oil triglycerides in SC-CO_2 as a function of temperature and pressure.

Fig. 9 Solubility of water in SC-CO_2 as a function of temperature and pressure.

Fig. 10 Effect of sample moisture content on the SC-CO_2 extraction of a smoked ham sample.

Fig. 11 Schematic of a supercritical fluid extraction device for collecting non-volatiles (R) as well as volatiles on a Tenax trap.

Fig. 12 Percent pesticide recovery through an extraction cell loaded with alumina for sample cleanup.

Fig. 13 Integration of sorbent collection device based on SPE cartridge, for both off- and in-line trapping.

Fig. 14 Comparison of packed column GC/ECD chromatograms of incurred pesticide residues in SFE extract from poultry adipose tissue: (A) supercritical fluid cleanup; (B) blank CO_2 collection (20 min) prior to injection; (C)fraction collected immediately following sample, 10 min collection time; (D) conventional cleanup methodology.

Fig. 15 Sequence of steps for inverse SFE vs. normal SFE.

Fig. 16 GC/ECD chromatogram of incurred pesticides extracted from chicken fat using fluoroform.

Fig. 17 Mechanism of lipase-catalyzed hydrolysis(left side) and alcoholysis (right side) of triglycerides.

Fig. 18 SFE apparatus with sorbent trap option: TP = cylinder pressure; RD = rupture disk; CF = check valve and filter; PG = pressure gauge; SV = switching valve; TC = thermocouple; MV = micrometering valve; GT = gas totalizer.

Fig. 19 Automated SFE/SFR/GC analyzer for the determination of fat content in foods. Legend: (A) cylinder; (B) methanol; © high pressure pump; (D) valve; (E) extraction cell - (1) sample, (2) glass wool plug, (3) supported lipase; (F) analyte trap; (G) hexane rinse solvent; (H) rinse solvent pump; (I) sample vial; (J) GC autoinjector tray; (K) gas chromatograph.

Fig. 20 Comparison of AOCS official method (G3-53) for fatty acid content of soapstock with results from SFE/SFR and SFC methods.

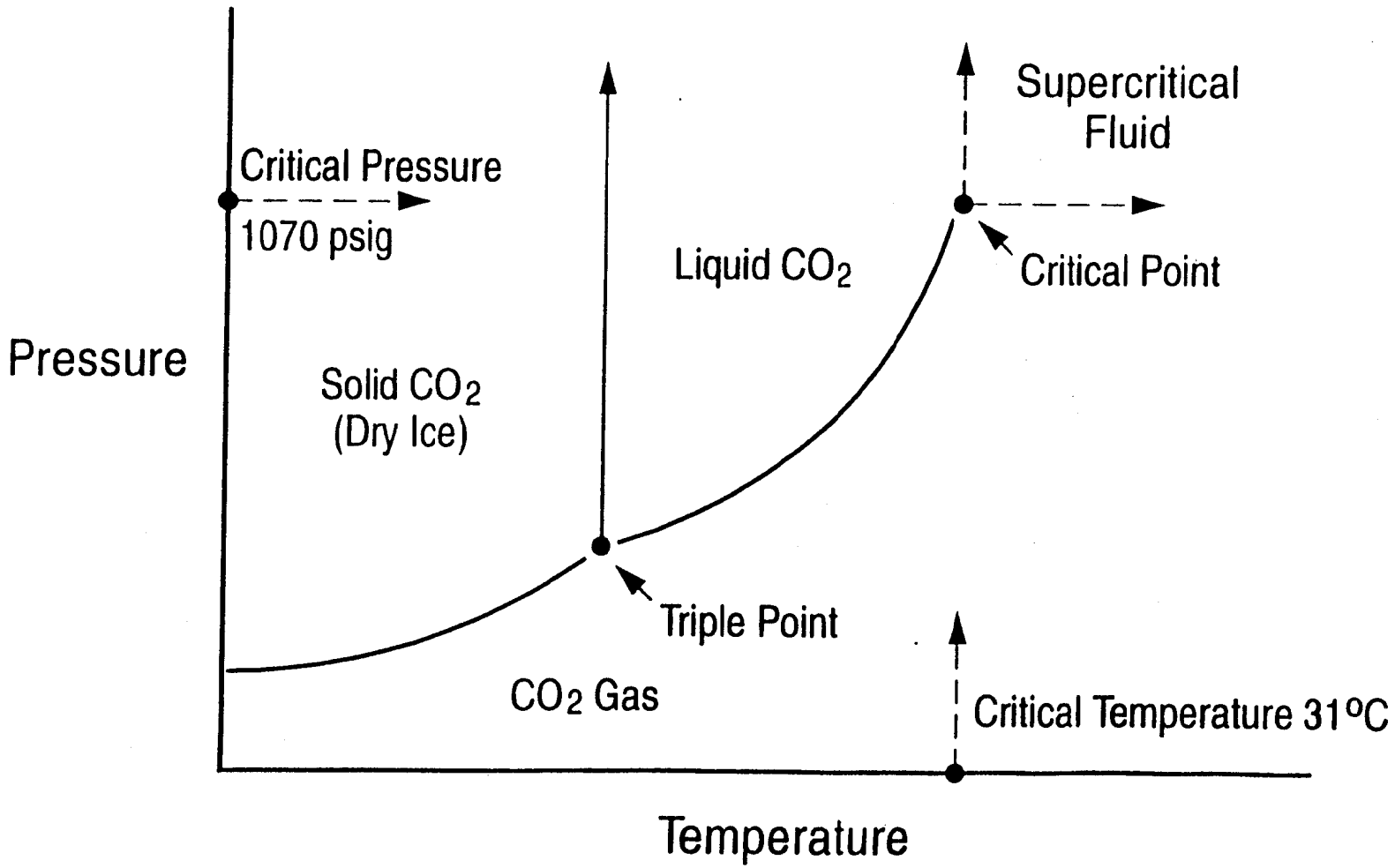
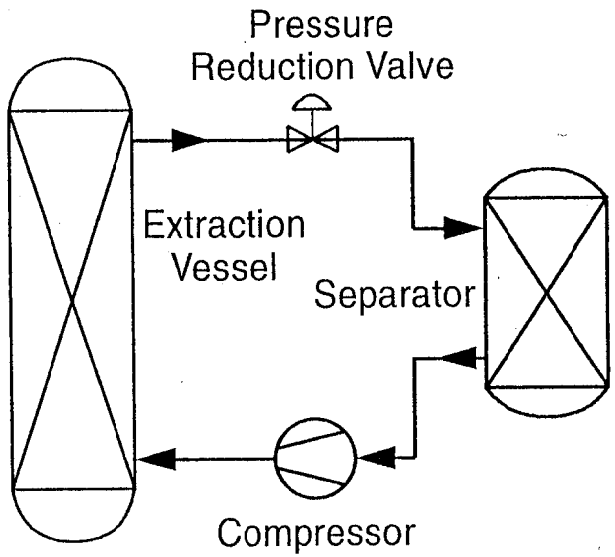

Supercritical
Fluid
Critical Pressure
1070 psig
Liquid CO_2
Critical Point
Pressure
Solid CO_2
(Dry Ice)
Triple Point
CO_2 Gas
Critical Temperature 31°C
Temperature

Fig. 2

Supercritical Fluid Extraction Process



Solubility of Naphthalene in CO_2

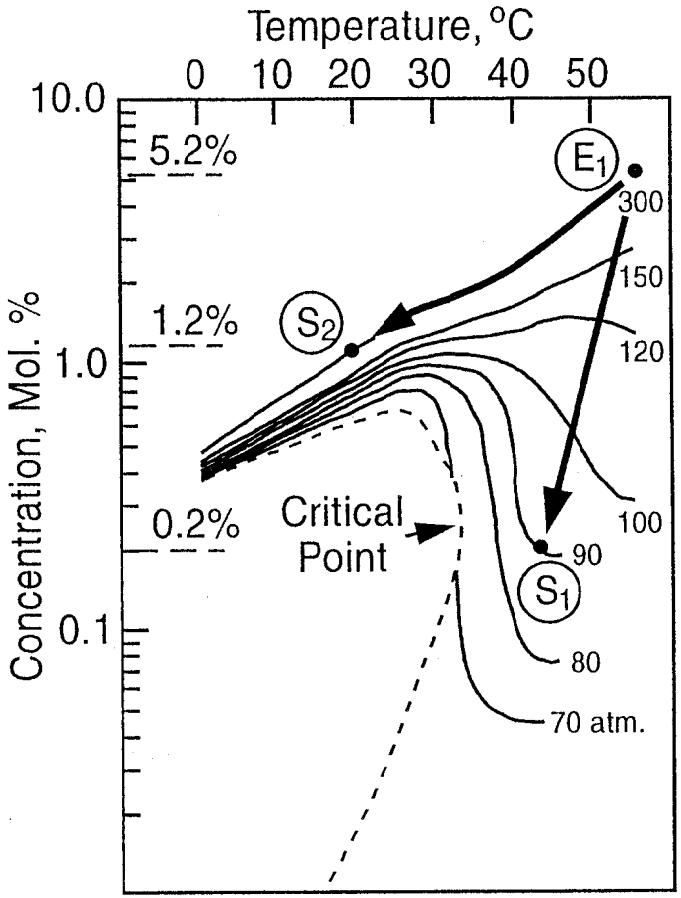
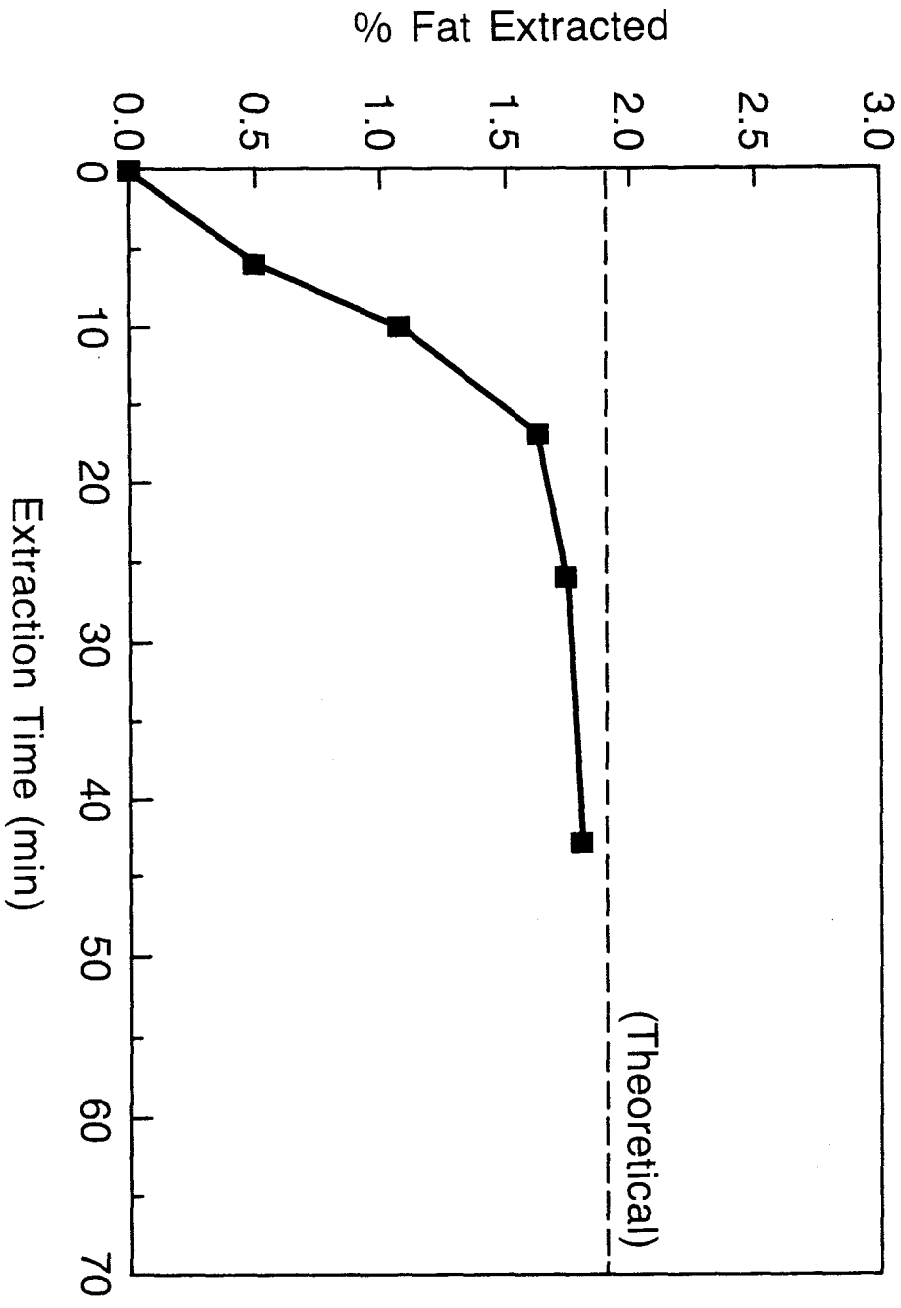
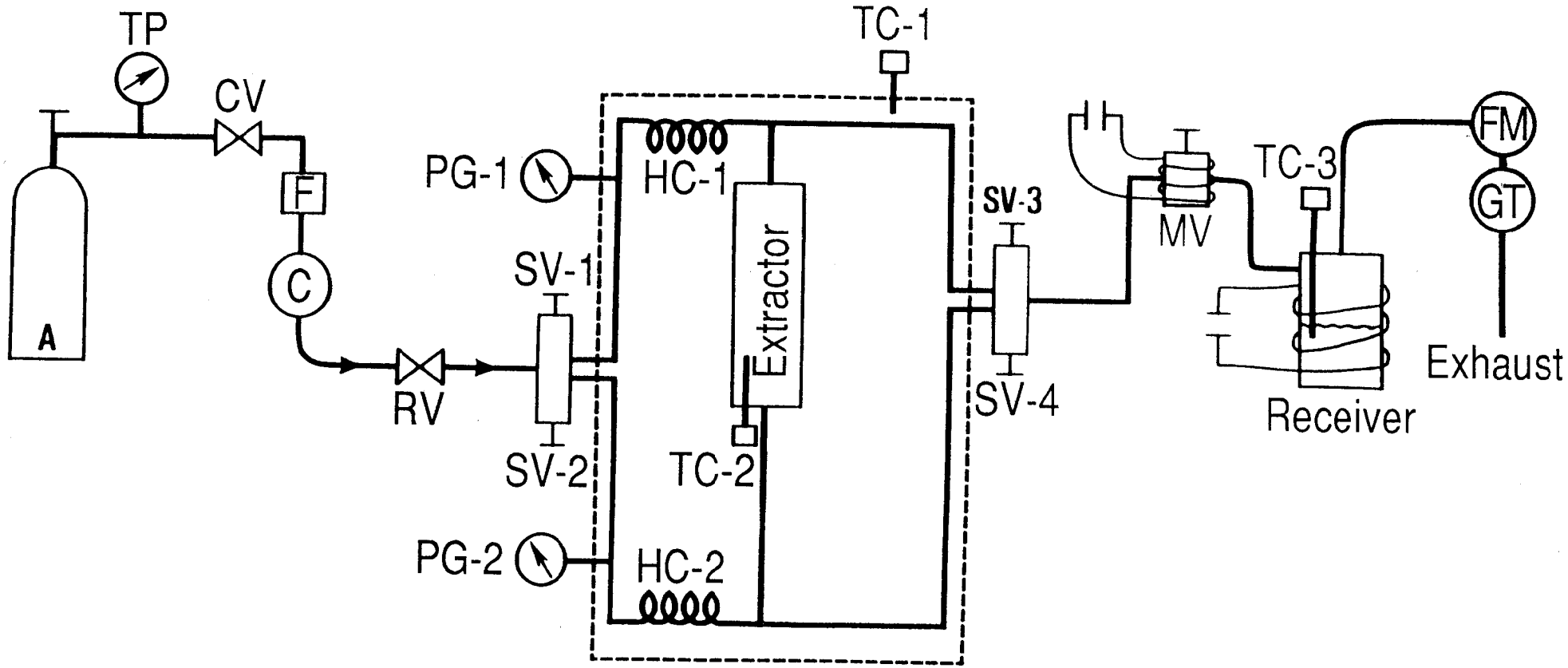
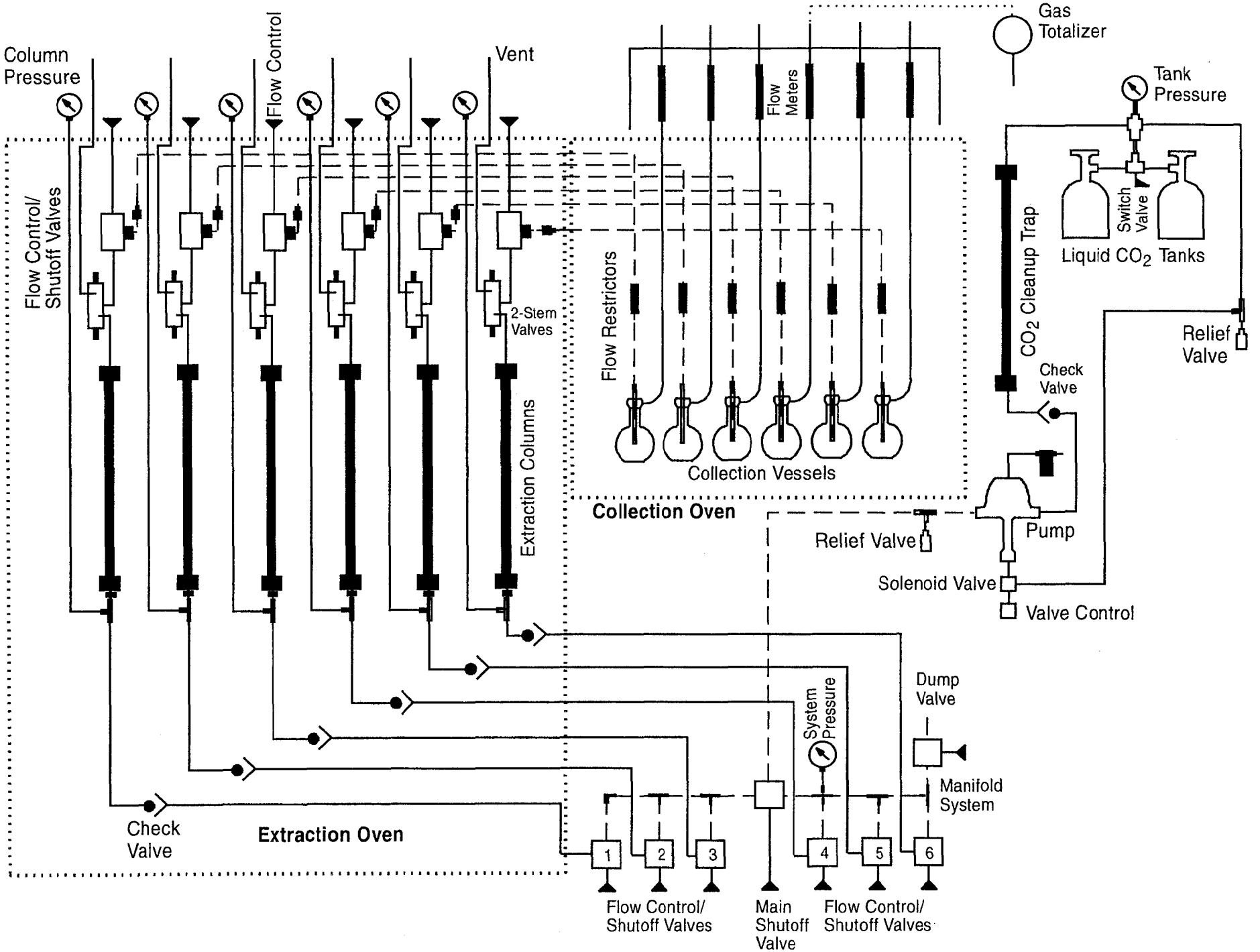


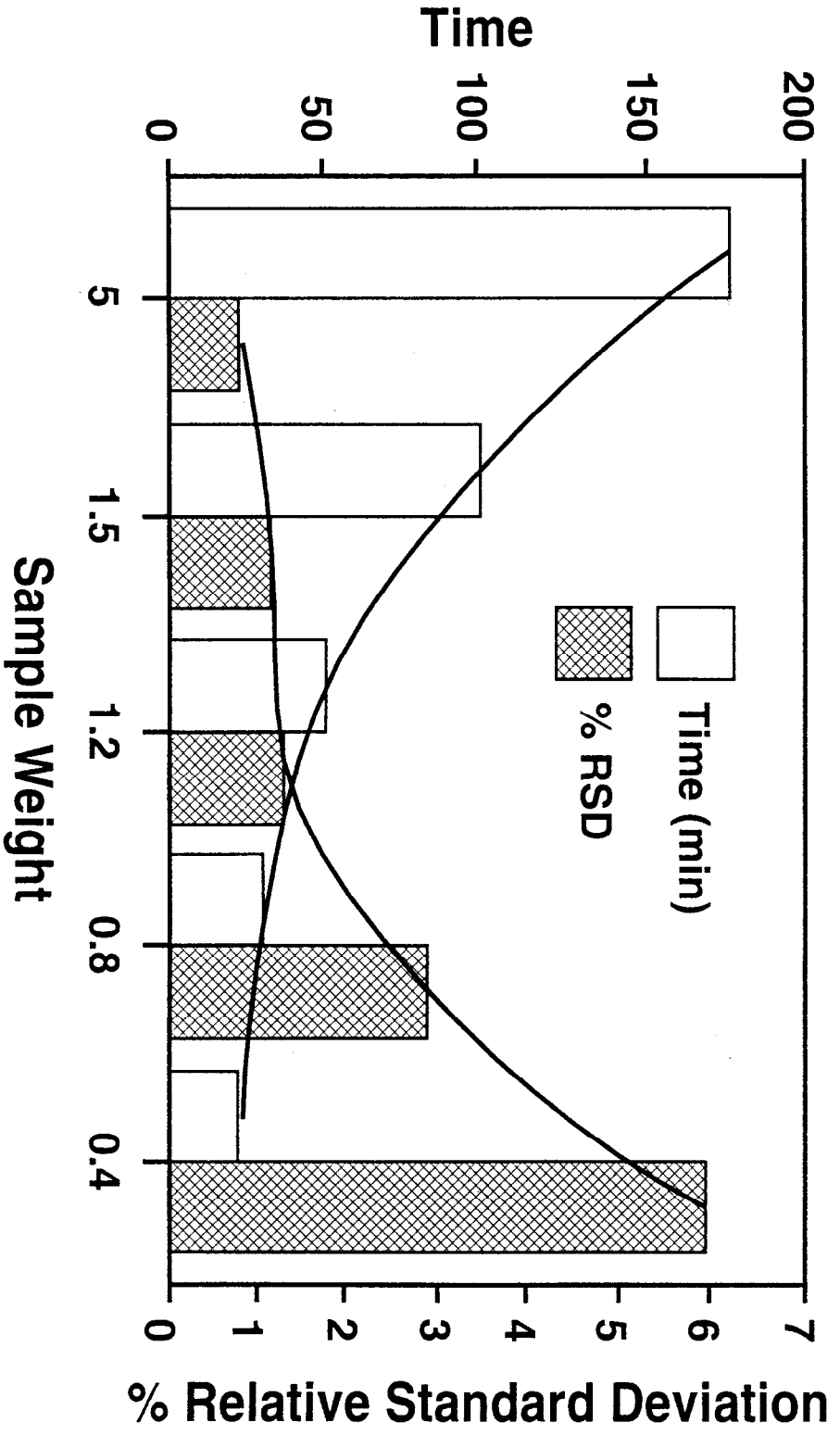
Fig. 2

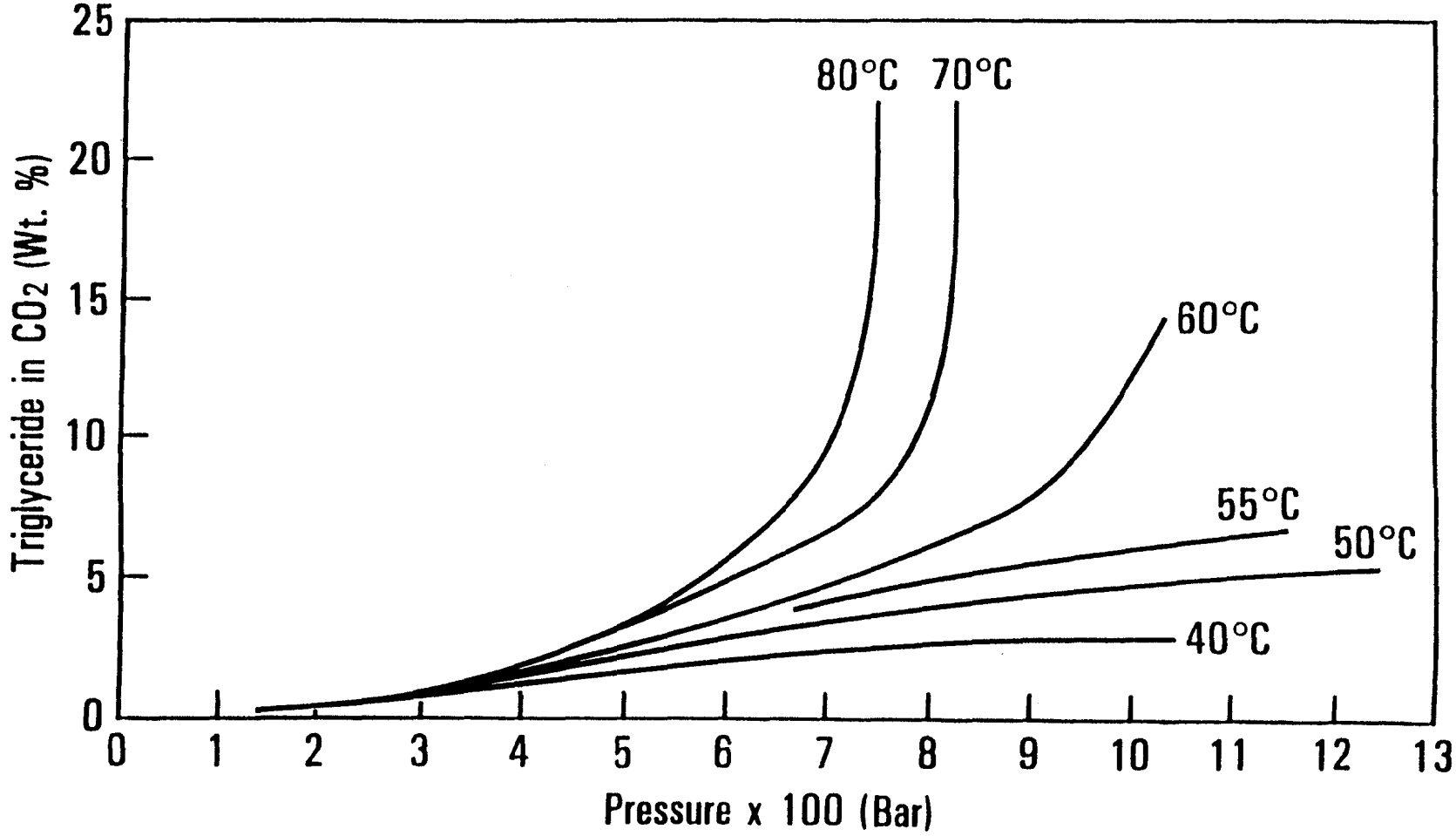
Fig. 3

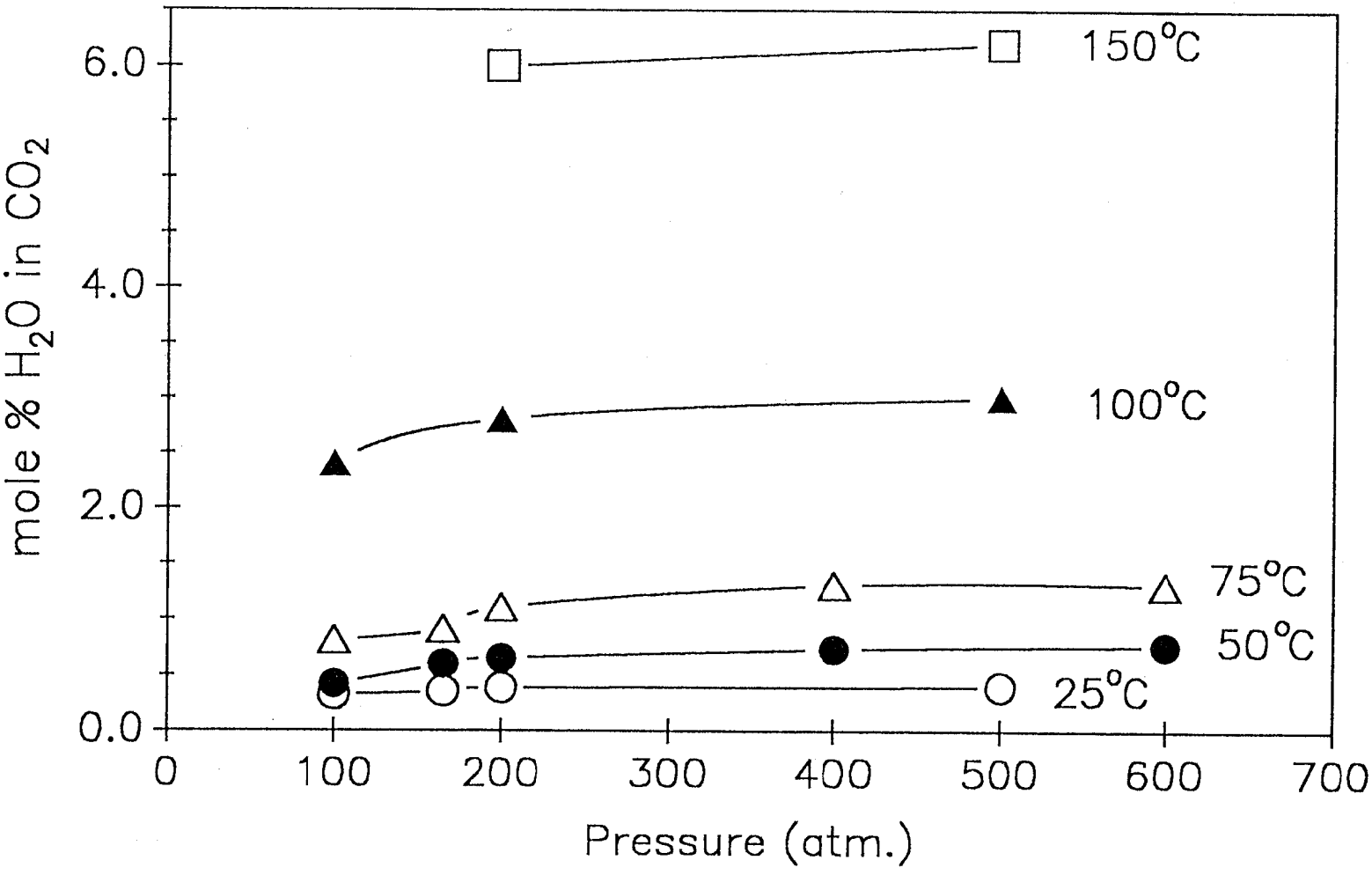

% Fat Extracted
0.0
0.5
1.0
1.5
2.0
2.5
3.0
(Theoretical)
0
10
20
30
40
50
60
70
Extraction Time (min)


TP
CV
A
F
C
RV
PG-1
SV-1
SV-2
PG-2
HC-1
HC-2
TC-1
Extractor
TC-2
SV-3
SV-4
MV
TC-3
Receiver
FM
GT
Exhaust

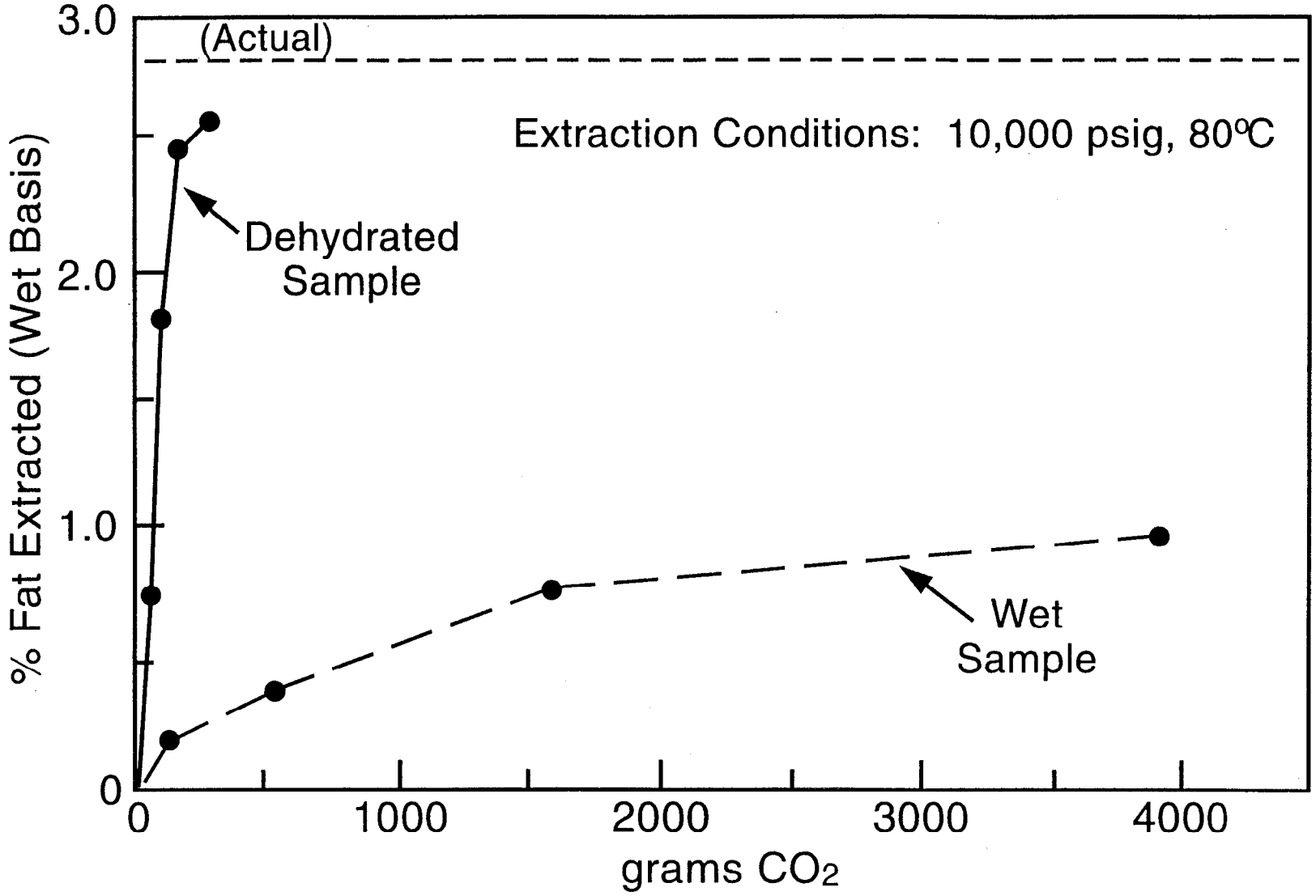

Column Pressure
Flow Control
Vent
Gas Totalizer
Tank Pressure
Flow Meters
Flow Control/ Shutoff Valves
2-Stem Valves
Switch Valve
Liquid CO_2 Tanks
CO_2 Cleanup Trap
Relief Valve
Flow Restrictors
Check Valve
Extraction Columns
Collection Vessels
Collection Oven
Relief Valve
Pump
Solenoid Valve
Valve Control
Dump Valve
System Pressure
Manifold System
Check Valve
Extraction Oven
1
2
3
4
5
6
Flow Control/ Shutoff Valves
Main Shutoff Valve
Flow Control/ Shutoff Valves

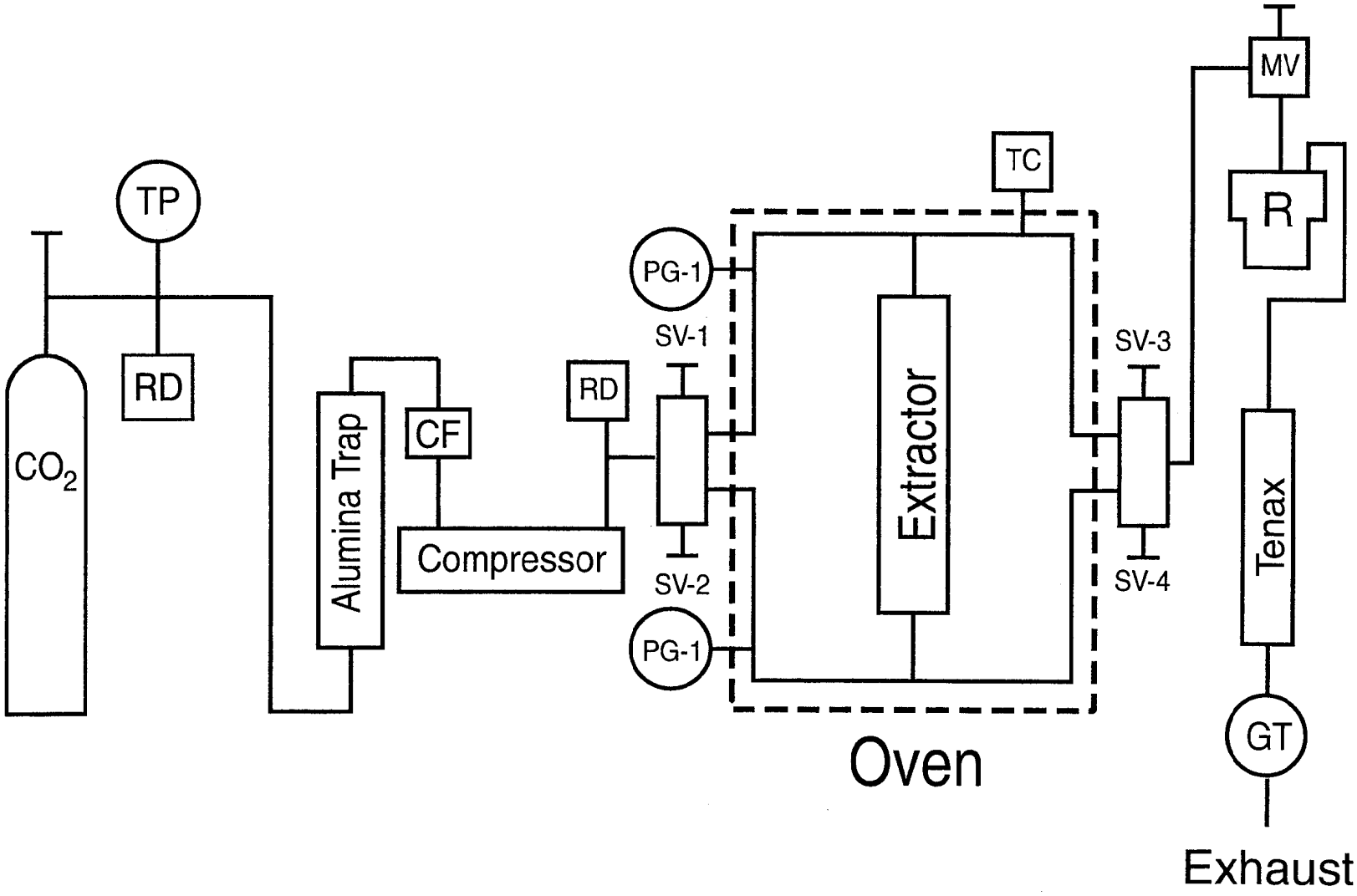

Time
0
50
100
150
200
Sample Weight
5
1.5
1.2
0.8
0.4
% RSD
Time (min)
0
1
2
3
4
5
6
7
% Relative Standard Deviation

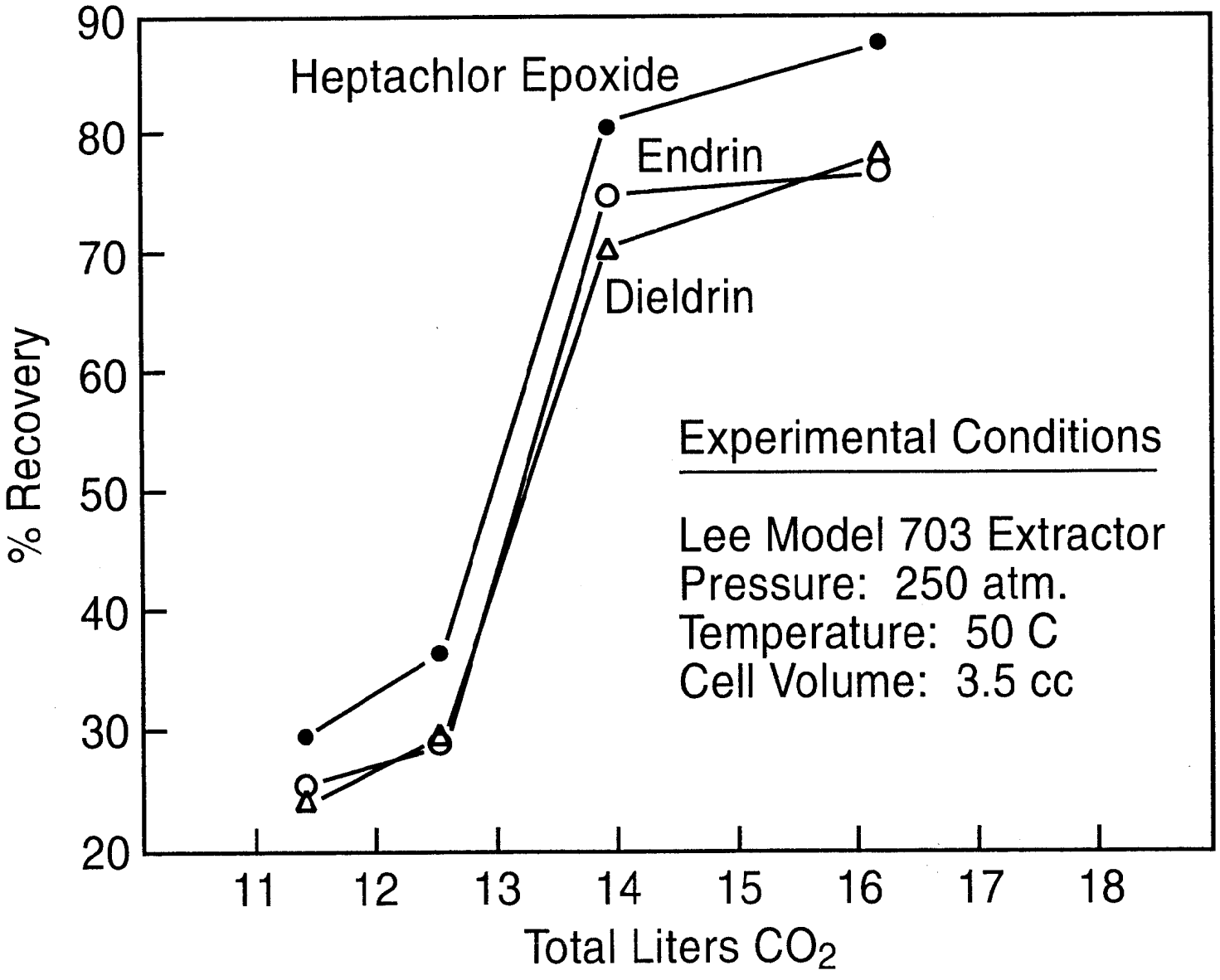

25
20
15
10
5
0
Triglyceride in CO_2 (Wt. %)
80°C
70°C
60°C
55°C
50°C
40°C
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Pressure x 100 (Bar)



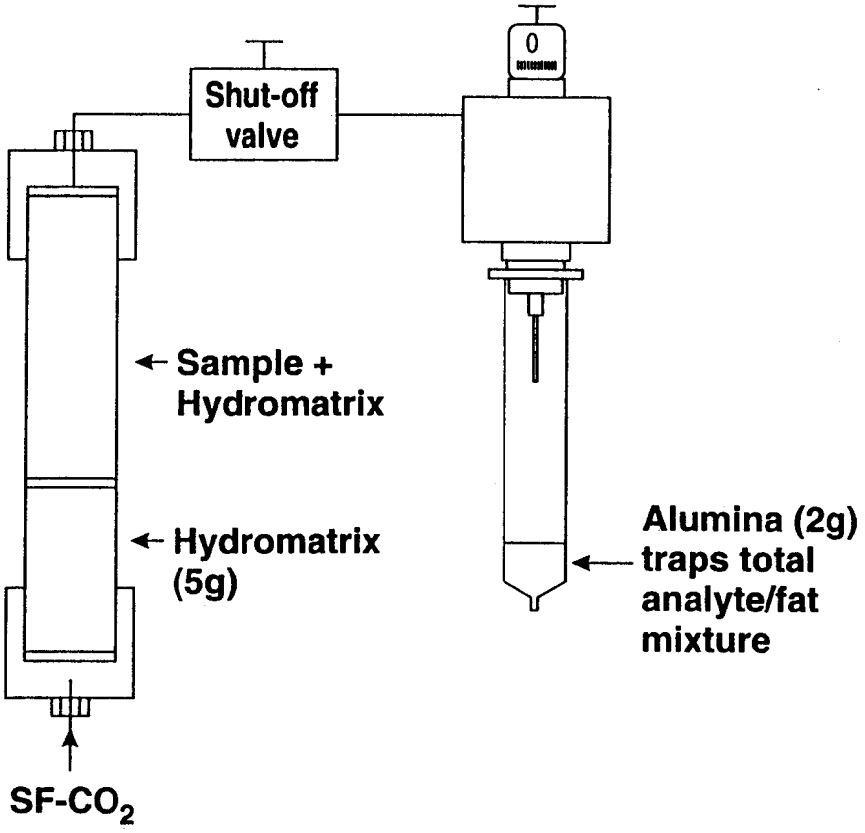
*Data from: K.A.Evelein et al., I&EC, Proc.Des.Dev.1976,15,423.


3.0
(Actual)
Extraction Conditions: 10,000 psig, 80°C
Dehydrated Sample
2.0
1.0
0
% Fat Extracted (Wet Basis)
Wet Sample
0
1000
2000
3000
4000
grams CO_2

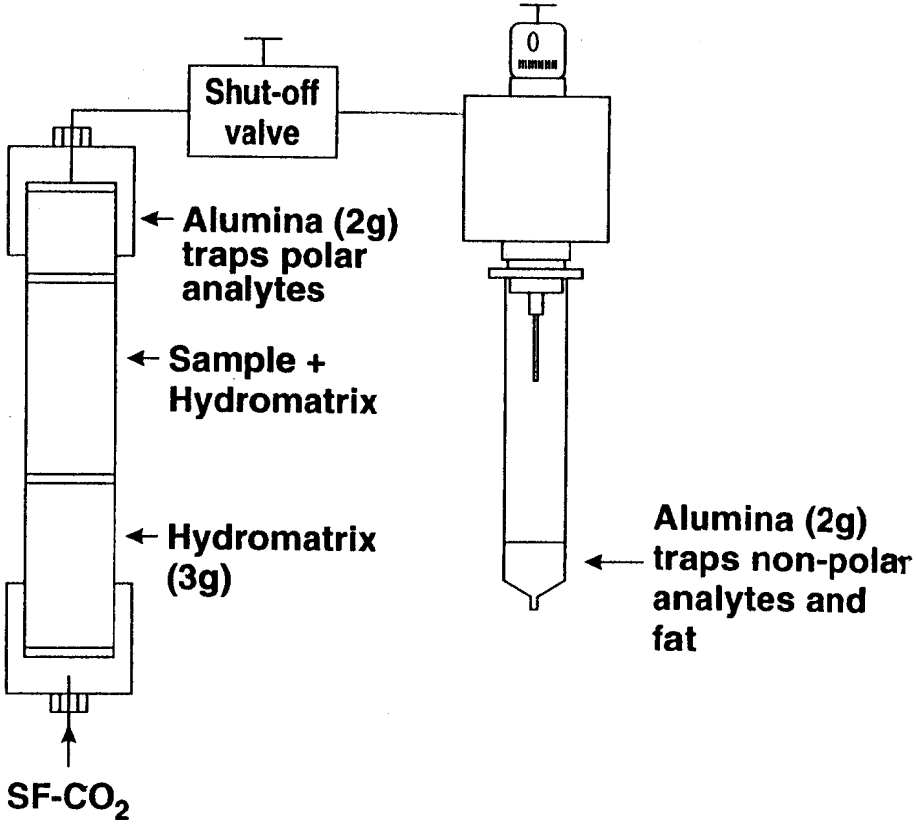

TP
RD
CO_2
Alumina Trap
CF
Compressor
RD
PG-1
SV-1
SV-2
PG-1
TC
Extractor
SV-3
SV-4
MV
R
Tenax
GT
Oven
Exhaust

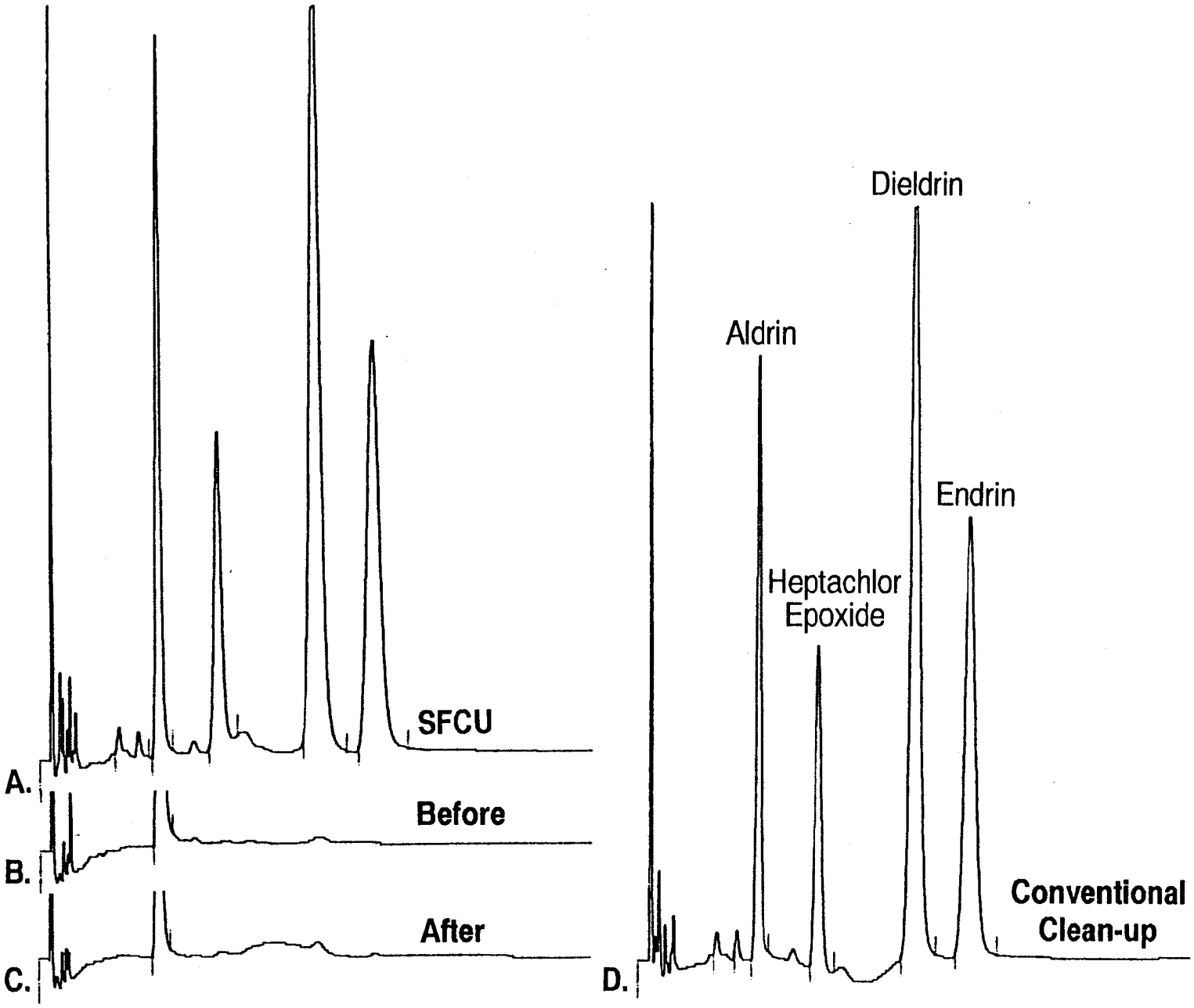

90
80
70
60
50
40
30
20
% Recovery
Heptachlor Epoxide
Endrin
Dieldrin
Experimental Conditions
Lee Model 703 Extractor
Pressure: 250 atm.
Temperature: 50 C
Cell Volume: 3.5 cc
11
12
13
14
15
16
17
18
Total Liters CO_2

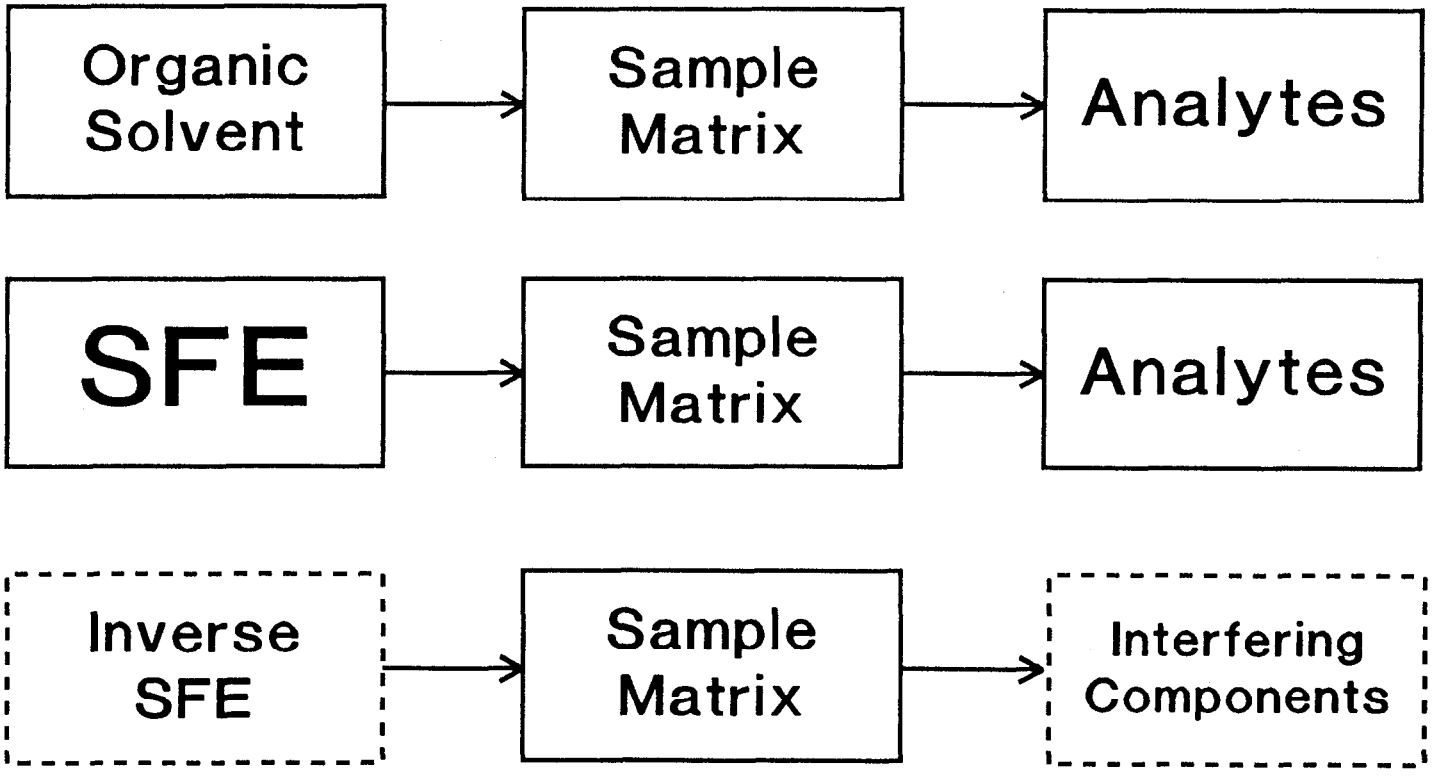
Off-Line Trapping

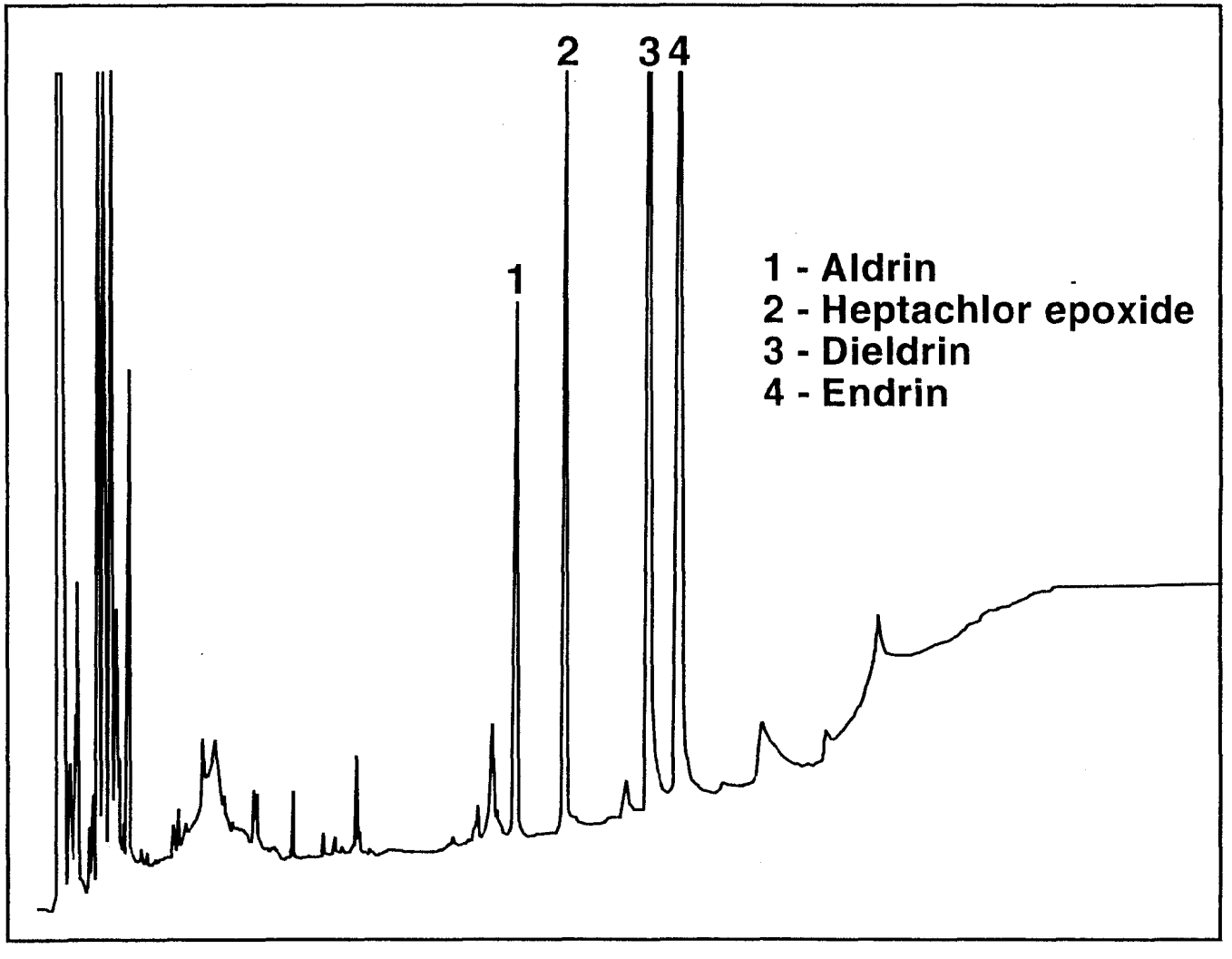


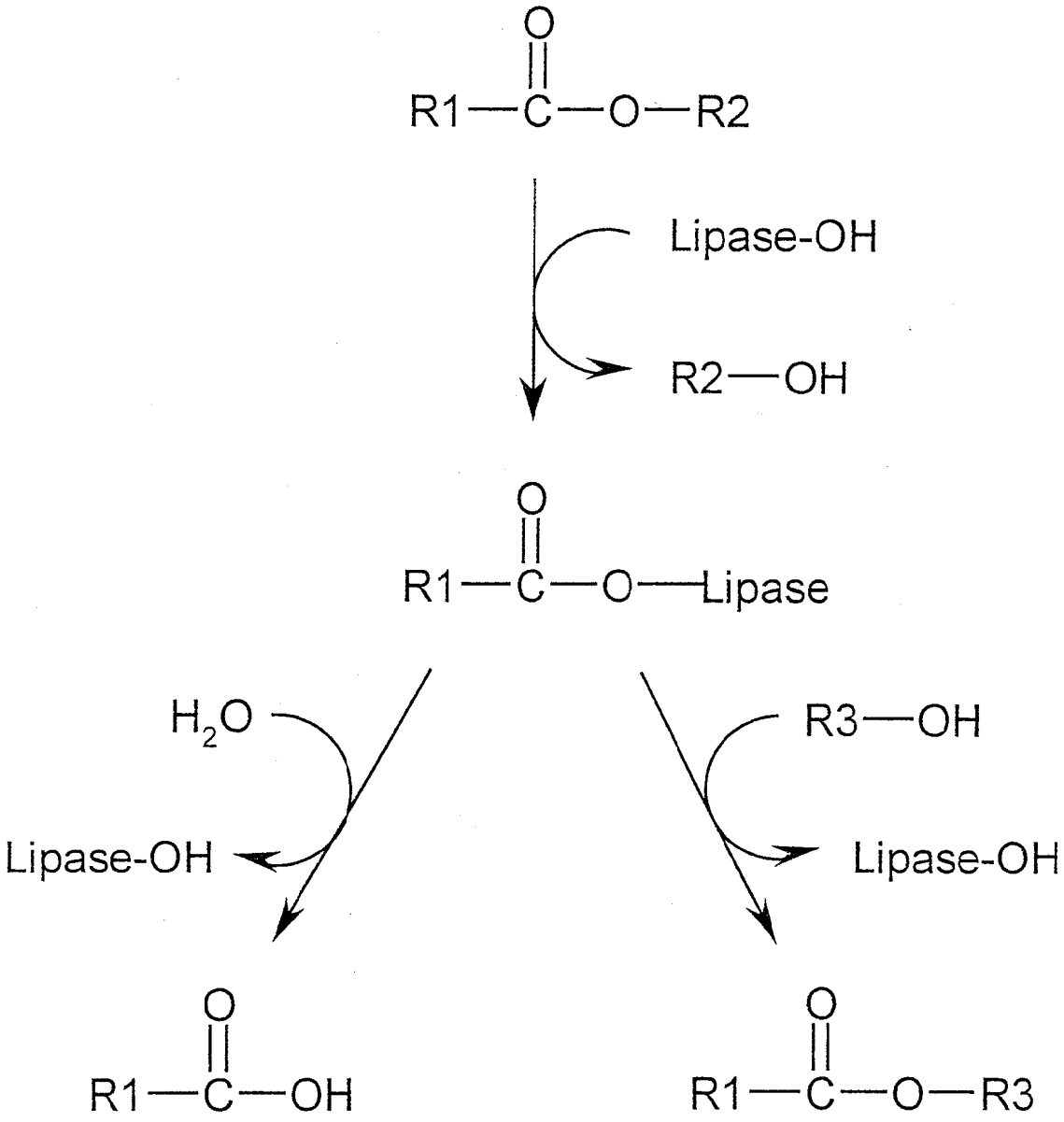
Tandem Off-Line & In-Line Trapping



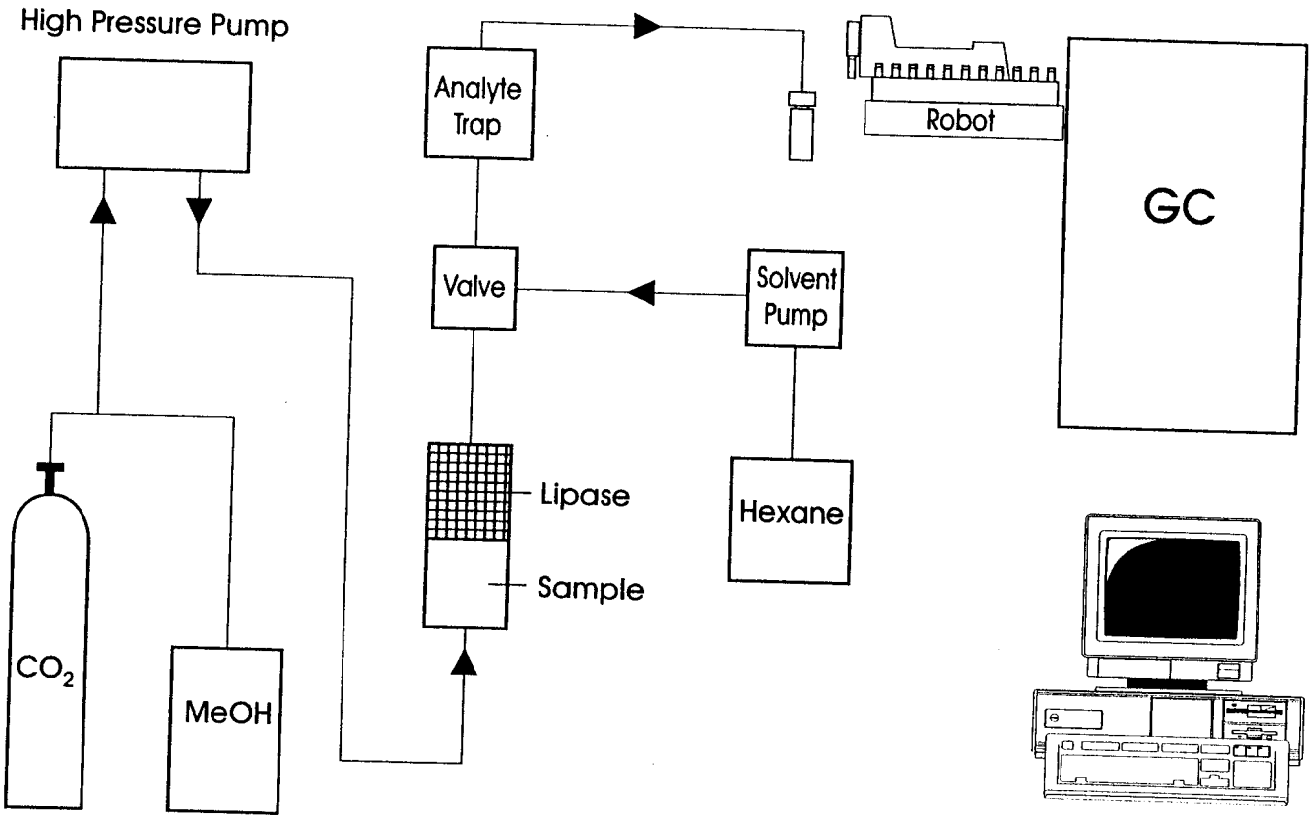

A.
SFCU
B.
Before
C.
After
D.
Aldrin
Dieldrin
Heptachlor
Epoxide
Endrin
Conventional
Clean-up

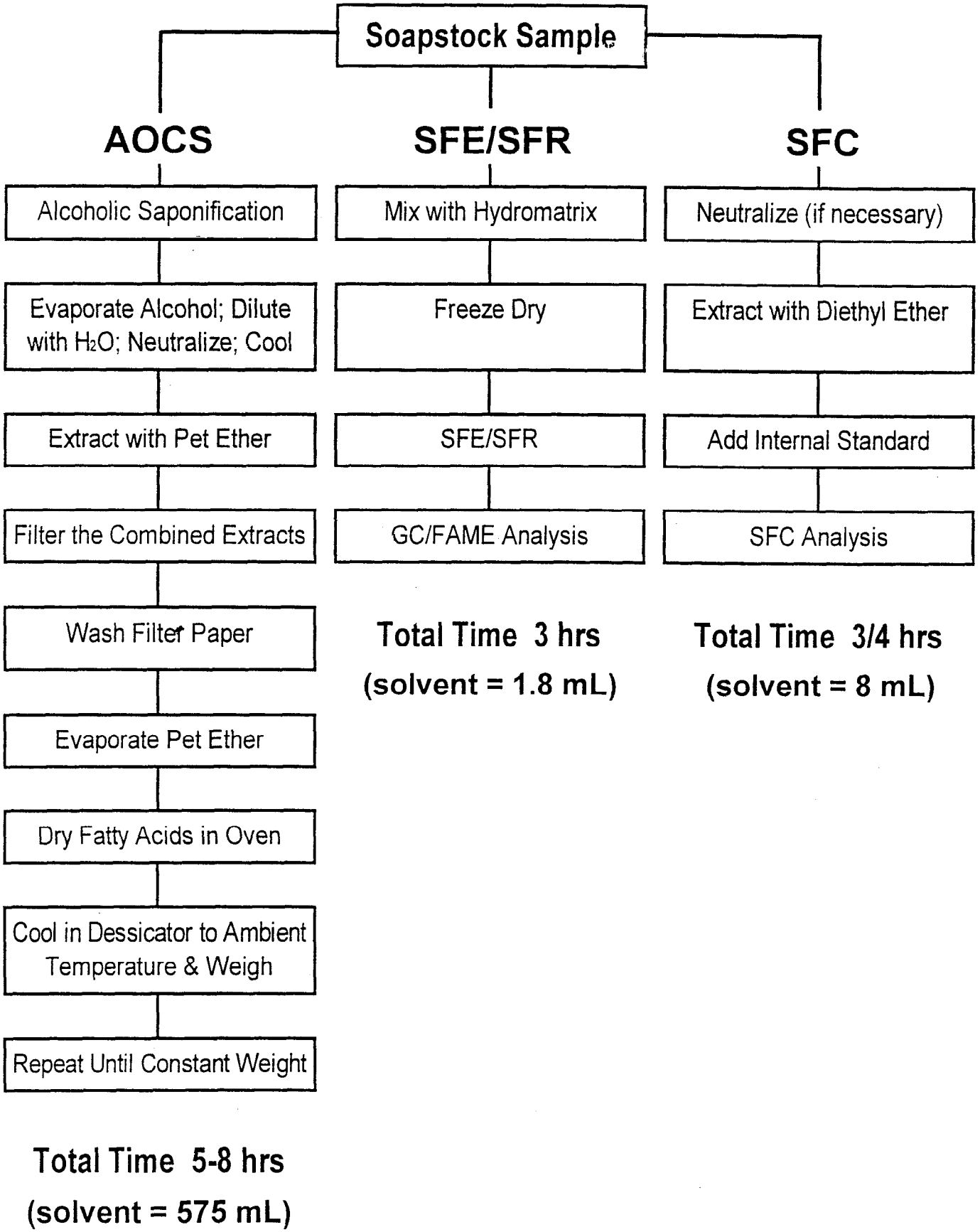

Organic Solvent
Sample Matrix
Analytes
SFE
Sample Matrix
Analytes
Inverse SFE
Sample Matrix
Interfering Components


1
2
3 4
1 - Aldrin
2 - Heptachlor epoxide
3 - Dieldrin
4 - Endrin


R1—C—O—R2
Lipase-OH
R2—OH
R1—C—O—Lipase
H2O
Lipase-OH
R3—OH
Lipase-OH
R1—C—OH
R1—C—O—R3

CO2
RD
TP
Alumina Trap
CF
COMPRESSOR
RD
PG-2
SV-2
SV-1
PG-1
OVEN
Extractor
TC
SV-4
SV-3
EXHAUST
GT
Florisil Trap
MV


High Pressure Pump
Analyte Trap
Robot
GC
Valve
Solvent Pump
Lipase
Sample
Hexane
CO_2
MeOH


Soapstock Sample
AOCS
SFE/SFR
SFC
Alcoholic Saponification
Evaporate Alcohol; Dilute with H_2O; Neutralize; Cool
Extract with Pet Ether
Filter the Combined Extracts
Wash Filter Paper
Evaporate Pet Ether
Dry Fatty Acids in Oven
Cool in Dessicator to Ambient Temperature & Weigh
Repeat Until Constant Weight
Total Time 5-8 hrs
(solvent = 575 mL)
Mix with Hydromatrix
Freeze Dry
SFE/SFR
GC/FAME Analysis
Total Time 3 hrs
(solvent = 1.8 mL)
Neutralize (if necessary)
Extract with Diethyl Ether
Add Internal Standard
SFC Analysis
Total Time 3/4 hrs
(solvent = 8 mL)